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Full Length Research Paper

Identification and utility of sequence related amplified polymorphism (SRAP) markers linked to bacterial wilt resistance genes in potato

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Bacterial wilt caused by *Ralstonia solanacearum* is one of the most economically important diseases affecting potato (*Solanum tuberosum*). It is necessary to develop more molecular markers for potential use in potato genetic research. A highly resistant primitive cultivated species *Solanum phureja* was employed to generate a F1 mapping population to perform the bulked segregant analysis (BSA) for screening and identifying of sequence related amplified polymorphism (SRAP) markers linked to the potato resistance to bacterial wilt. A linkage map containing 23 DNA markers distributed on three linkage groups, and covering a genetic distance of 111 cm with an average distance of 5.8 cm between two markers was developed. Two SRAP markers, Me2em5 linked in repulsion phase and Me2em2 in coupling phase, flanked the resistance genes at genetic distances of 3.5 and 3.7 cm distance, respectively. These markers and two others were used for early seedling selection in a BC1 population. The results show that this marker system could be used in marker assisted selection (MAS) breeding program.

Key words: Sequence related amplified polymorphism (SRAP) marker, potato, bacterial wilt.

INTRODUCTION

Bacterial wilt caused by *Ralstonia solanacearum* is one of the most widely spread and very destructive plant disease, causing enormous economic losses. The vascular pathogen enter and colonize the plant vascular (xylem) system, disrupting water transport, and causing the characteristic symptoms of wilting, and often vascular discoloration, and death of aerial tissues (He, 1983; Hayward, 1991). The bacterial parasite cause diseases on over 400 plant species, including many crops such as potato, tomato and eggplant. The severity of attacks by this soil-

borne vascular parasite is known to vary considerably according to climate, farming practices, soil type and geographic locations (Guidot et al., 2007). Control of wilt diseases is also complicated by the scarcity of sources of disease-resistant host germplasm, and the soil and vascular habitats of the pathogen (Hayward, 1991; Esposito et al., 2008). Despite decades of interests in the pathology, epidemiology and control of bacterial wilt, very little is known about the sources and resistance of the disease and about the genetic or molecular mechanisms under-

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lying host plant resistance (Qu, 1996). Some nuclear SSR alleles derived from the wild species *S. chacoense* appeared to be related to bacterial wilt resistance (Chen et al., 2013).

The mode of inheritance of resistance in some tetraploid primitively cultivated species is not yet clearly known. Therefore, it is difficult to select bacterial wilt resistant clones or varieties in potato breeding. Although, method of biological control could be used in some situations to combat the pathogens, development of resistant cultivars is usually the best agronomic solution. Disease resistance breeding has traditionally been done by phenotypic selection. Efficiency of phenotypic selection is reduced by variability in the pathogen, infection and disease development. Molecular markers tightly linked to the resistance genes can eliminate these sources of phenotypic variation to enable more efficient breeding strategies in potato improvement (Li et al., 2013; Virupaksh et al., 2012). Marker-assisted selection can be helpful to bacterial wilt resistance breeding. We have identified some molecular markers like AFLP and RAPD markers that linked to potato resistance, these markers were found not to be effective to screen a large population containing up to 200 true potato seeds in the actual application (Gao et al., 2002, 2005). To accelerate introgression of the resistance into cultivated lines, it would be desirable to have more and better molecular markers to perform selection of those possible resistance genes.

Sequence-related amplified polymorphism (SRAP) technology has been recognized as a new and useful molecular marker system for mapping and gene tagging in many crops plants (Valdez-Ojeda et al., 2008; Cao et al., 2011; Niu et al., 2011; Guo et al., 2012; Deng et al., 2013). They are also useful in positional cloning of genes and in elucidating the genetic mode of complex traits that do not display Mendelian segregation (Levi, 2002). Marker-facilitated selection would be particularly effective for pyramiding more resistance genes to provide effective and potentially stable resistance to disease (Martin et al., 1991).

The objectives of this study were to determine the feasibility of using SRAP markers to develop a molecular linkage map of the *S. tuberosum*, and to identify marker loci associated with bacterial wilt. Both of these objectives were met, and we identified the SRAP markers that proved useful in selecting for the gene conferring bacterial wilt resistance in a BC1 population. A longer-term goal of this project will be to use closely linked molecular markers to introduce these resistant loci into potato cultivars as a potential strategy to control potato bacterial wilt disease in the field. In this study, the set of markers provide an impactful marker combination for use in a marker assisted selection (MAS) breeding program to identify genotypes containing resistance from original potato cultivars. Taken together, this study provides and sheds light into potential directions for development of novel management strategies for molecular breeding to

controlling wilt diseases.

MATERIALS AND METHODS

Plant materials

One genotype ED13, derived from the cross hybridization between 772102.37 (resistant) and USW7589.2 (susceptible) collected both from the Department of Plant Breeding, Wageningen University, the Netherlands was used as the resistant parent. The susceptible parent was derived from USW5337.3 × *Solanum phureja* (provided by Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, China). A segregating F1 population (Figure 4A) composed of 230 seedlings from hybrid true potato seed (TPS) was used for mapping the SRAP markers. Another segregating BC1 population were generated by first backcrossing a CE26 (susceptible, derived from a cross USW 5337.3 × 772102.37) with the parent 772102.37 for assessing the utility of candidate SRAP markers in identifying bacterial wilt-resistant BC1 plants (Figure 4B).

Wounded root plus soil inoculation

Inocula for potato plants at a concentration of 1×10^8 cfu/ml were made from cultures grown on BPG plates at 28°C for 48 h using SMM without agar and were incubated at 28°C for 4 h. Potato plants with six to eight fully expanded leaves were inoculated by pouring 50 ml of bacterial suspension into the soil around the base of the stem with wounding the roots with a knife. 15 potato seedlings were inoculated with the strain PO41 of the bacterium (provided by the Institute of Plant Protection, Chinese Academy of Agricultural Sciences, China). The virulent, wild colony type of *R. solanacearum* cultures was selected on 2, 3, 5-tetrazolium chloride medium (TZC) and used for inoculation. Inoculated plants were cultivated at 28°C under a 16/8 h (light/dark) photoperiod in a growth chamber and were not watered five days before and after inoculation. Control plants were mock-inoculated with sterile water. Each treated plant was rated daily for disease for 21 days after inoculation. Symptoms were scored daily on a 0 to 5 disease index, where 0 indicates no disease, 1 indicates 1 to 10% of leaves wilted, 2 indicates 10 to 25% of leaves wilted, 3 indicates 25 to 50% of leaves wilted, 4 indicates 50 to 75% of leaves wilted, and 5 indicates 75 to 100% of leaves wilted. Each experiment contained 16 plants per treatment, and experiments were repeated at least three times. Leaf tissues of the susceptible were sampled 4 to 7 dpi into liquid nitrogen for DNA extraction, while the resistant samples were collected 8 to 14 dpi due to slower disease development in this resistant host.

Sequence-related amplified polymorphism analysis

DNA was isolated according to Ducreux et al. (2008). Bulk segregant analysis (BSA) (Michelmore et al., 1991) was used to identify SRAP markers linked to the bacterial wilt resistance gene. For BSA, resistant and susceptible DNA bulks were composed of 10 most resistant (disease index 0) plants and most susceptible (disease index 5), respectively. SRAP analysis was conducted according to previously established protocols with minor modifications (Li et al., 2001). The PCR reaction was set up in a final volume of 20 µL containing 50 ng of DNA, 5.0 pmol of primer, 200 mM dNTPs, 1.5 mM MgCl₂, and 0.5U of Taq polymerase (Sangon Biotech Co. Ltd., Shanghai, China) in 1× Taq buffer. The PCR program included an initial denaturing at 94°C for 3 min followed by 8 cycles of 94°C for 30 s, 37°C for 30 s, and at 72°C for 90 s and then 35 cycles of 94°C for 30 s, 48°C for 30 s, and 72°C for 90 s with a final extension of 72°C for 10 min. PCR products were separated using 8% denaturing polyacrylamide gel electrophoresis and visualized by fast silver staining (Bassam et al., 1991).

Table 1. The primer sequences of SRAP used in this study.

Forward primer		Reverse primer	
me1	TGAGTCCAAACCGGATA	em1	GACTGCGTACGAATTAAT
me2	TGAGTCCAAACCGGAGC	em2	GACTGCGTACGAATTTGC
me3	TGAGTCCAAACCGGAAT	em3	GACTGCGTACGAATTGAC
me4	TGAGTCCAAACCGGACC	em4	GACTGCGTACGAATTTGA
me5	TGAGTCCAAACCGGAAG	em5	GACTGCGTACGAATTCGA
me6	TGAGTCCAAACCGGTAA	em6	GACTGCGTACGAATTAAC
me7	TGAGTCCAAACCGGACG	em7	GACTGCGTACGAATTGCA
		em8	GACTGCGTACGAATTCAA

The primer combinations (Table 1) that generated polymorphic bands between the bulks were tested on the bulked individuals to eliminate false-positive markers.

Linkage analysis of markers

Several markers obtained previously were employed before the construction of linkage map to increase reliability; those include three AFLP, one RAPD markers. Preliminary screening for candidate seedling was carried out on the two populations using these markers. As expected, more than 80% of the individual genotype was detected to contain all or one relevant marker DNA fragment. Based on the preliminary selection, Linkage analysis was performed with the JoinMap software (Van Ooijen and Voorrips, 2001). Prior to linkage analysis segregation rates, SRAP fragments that ranged in size of 75 to 500 bp were scored for marker. Presence or absence of each polymorphic fragment was coded as "1" and "0", where "1" indicated the presence of a specific allele, and "0" indicated its absence. For each segregating marker, a chi-square test was performed to fit for deviation from the 1:1 expected segregation ratio in a testcross or BC1 population under the 'locus genotypic frequency' command. Markers were sorted on the basis of the chi-squares test with a P -value of <0.05; and skewed markers were excluded from the analysis. All markers were analyzed for linkage, and recombination fractions were converted into map distances (centimorgans) while employing the Kosambi mapping function.

Logarithm of the odds ratio for linkage (LOD) scores of 2.0 to 6.0 were used for grouping of markers, followed by high threshold LOD scores of 7.0 to 10.0 for final mapping of markers in each linkage group. Loci showing weak or suspect linkages were removed from the analysis.

Assessing the utility of a marker set in identifying bacterial wilt-resistant BC1 plants

Forty-one (41) BC1 plants were generated by first backcrossing bacterial wilt-susceptible CE26 genotype with the recurrent parent 772102.37. The marker set identified here were used to select for those progeny that presumably contain the corresponding DNA allele conferring bacterial-wilt resistance, and then three times strict phenotypic identification (the disease index was scored for wilt symptoms as described earlier) were employed to exanimate their identical degree. Spearman correlation coefficient was used to analyze the correlation between the molecular markers and the disease parameters statistical software package S-Plus Version 6. Probabilities <0.01 were considered as significant. All p values were based on two-sided tests, and the differences were considered statistically significant when the p value was ≤ 0.05 . All molecular detection and phenotypic identification were repeated three times.

RESULTS

Polymorphism and screening of SRAP markers

100 primer combinations were first tested for selective amplification of DNA fragments from the resistant and susceptible bulks to screening the polymorphism in the diploid mapping population. Prior to linkage analysis segregation rates, SRAP fragments that ranged in size of 75 to 500 bp were scored for marker. Fifty-six (56) of the polymorphic SRAP primer combinations amplified inconsistent band patterns per line. This inconsistency may have been the results of residual heterozygosity or the amplification of similar sequences in two separate genomic regions. It may also result from the use of polyacrylamide gels, which have a higher resolution power than most agarose gels. A total of 314 unambiguous bands were amplified by the 38 of 54 SRAP primer combinations, of which 187 bands were polymorphic (59.55%) and ranged in size from 50 to 1000 bp. The number of polymorphic fragments for each SRAP primer combination varied from 2 to 15, with an average of 6.0 fragments per primer combination. Polymorphisms were amplified with 38 primer pairs (67.8%) resulting in 146 polymorphic bands between the resistant and susceptible parental genotypes. Based on these results, primer pairs that generated polymorphic bands were tested on the resistant and susceptible bulks.

SRAP bands present in one pool and absent in the other were regarded as candidate markers linked to bacterial wilt resistance. SRAP fragments that ranged in size of 75 to 500 bp were scored for marker. A relatively small number of these primer combinations (4 of 54 pairs; 7.41%) were not suitable for the mapping experiment within the tested population because of the lack of polymorphism in size of 75 to 500 bp. Hence, out of the original 56 primer pairs, only 30 combinations were used. However, three (5.56%) of these combinations were skewed from the expected 1:1 segregation ratio and had to be excluded from the final linkage analysis. Twenty-seven (27) primer combinations produced 86 SRAP candidate markers that were used in the next linkage analysis.

Identification of SRAP markers linkage map

In order to obtain more closely linked markers and to avoid any possible mapping errors, the stringent linkage analysis criteria used with the JoinMap analysis (Van Ooijen et al., 2001) resulted in linkage groups. We compared for each primer combination, all primer pairs that generated polymorphic bands were tested on the resistant and susceptible bulks. SRAP bands present in one pool and absent in the other were regarded as candidate markers linked to bacterial wilt resistance. These SRAP markers were uniquely present in one of the donor parents and in the F1 individual genotypes. Although, a total of eighty-six SRAP markers were suitable for the mapping analysis using the first filial generation population, 23 (26.7%) of these markers were skewed from the expected 1:1 segregation ratio and had to be excluded from the final linkage analysis. Sixty-three (63) SRAP markers were used in the final linkage analysis. Five markers (5.8%) did not show strong linkage to our aim, and they were excluded from the analysis. A total of 58 SRAP markers were analyzed for linkage. Of these, 23 could be mapped with high confidence on the linkage map, four markers were clustered on linkage group 1, and seven markers were on linkage group 2 and twelve on linkage group 3 (Figures 1 and 2).

Markers linked in coupling or repulsion phase

Among the twelve polymorphic bands on linkage group 3, we found four markers tightly linked to the resistance locus, two of them linked in coupling phase and the others linked in repulsion phase. The primer combinations me2em5 and me5em2 (Table 1) were respectively detected, a band polymorphic between the resistant bulk and the susceptible bulk, since the marker band named as Me2em5 and Me5em2 was present in all of the susceptible plants in the bulks. They were screened out from the F1 population segregating for bacterial wilt resistance. When the phenotype identification was performed, this marker also co-segregated with disease reaction in the same fashion. And the linkage was further tested in the BC1 plants. The band was present in most of the susceptible genotypes and absent in the resistant ones. These results indicated a tight linkage between the marker me1em2 and the dominant susceptibility allele in the repulsion phase. The markers linked in repulsion phase exhibited similar electrophoretic images (Figures 2 and 3); While the primer combinations me2em2 and me5em1 detected approximately two close-migrating concomitant polymorphic bands between the resistant and susceptible individuals (Figure 2). These double-banded marker linked in coupling phase were designated Me2em2 and Me5em1, respectively.

The two polymorphic bands appeared in most of the resistant genotypes of the F1 population, and of which this proportion basically fits the expected 1:1 ratio, after

all, as there are some medium-resistant or medium-susceptible genotypes in the population detected by phenol-type identification. As expected, the band types in the gel were present reproducibly in the BC1 population (Figure 3).

Practical utility of the markers

In previous studies, several markers including AFLP, RAPD and SSR markers were described to link with bacterial wilt resistance loci, but did not determine their usefulness well in MAS programs. Here, we tested 41 BC1 plants inoculated with the pathogen for the presence or absence of the new SRAP markers. Four markers were used to select for those progeny that presumably contain the corresponding DNA allele conferring bacterial-wilt resistance following three times strict phenotypic identification (Figure 3). As shown in Figure 3A, of the 17 plants whose disease index were rated as 0 (highly resistant), 11 were detected to contain all four markers as homozygous genotypes, six plants were heterozygous for the four markers, in which five plants were detected to contain three markers, while one plant contained only one SRAP marker. Of the 15 plants whose disease indexes were rated as 5 (highly susceptible), 14 were detected to contain the repulsion marker. These results suggest that there is at least one major locus in the four marker alleles, because there were one or two markers detected in other nine plants whose disease indexes showed mildly resistant or mildly susceptible. These results were consistent with the expectation.

DISCUSSION

Although, SRAP markers are feasible to generate polymorphic (Levi et al., 2006; Poczai et al., 2013), a relatively small number of these primer combinations were not suitable for the mapping experiment within the tested population because of the lack of polymorphism in size of 75 to 500 bp. Hence, out of the original 56 primer pairs, only 30 combinations were used. However, three (5.56%) of these combinations were skewed from the expected 1:1 segregation ratio and had to be excluded from the final linkage analysis. These SRAP markers proved to be enough efficient and reliable in the mapping analysis in this study. We initially used bulk segregant analysis (BSA) strategy (Michelmore et al., 1991) to identify SRAPs. BSA has been widely used in many crop species for detecting markers linked to genes conferring disease resistance (Hyten et al., 2009) and is a powerful method for identifying molecular markers that show association with a gene of interest or a specific region of the genome (Ren et al., 2012; Salinas et al., 2013).

Segregation distortion is widespread in plant populations and is a common feature of plant genetic linkage maps; it is frequent in progeny derived from interspecific crosses and distortion tends to increase with increasing

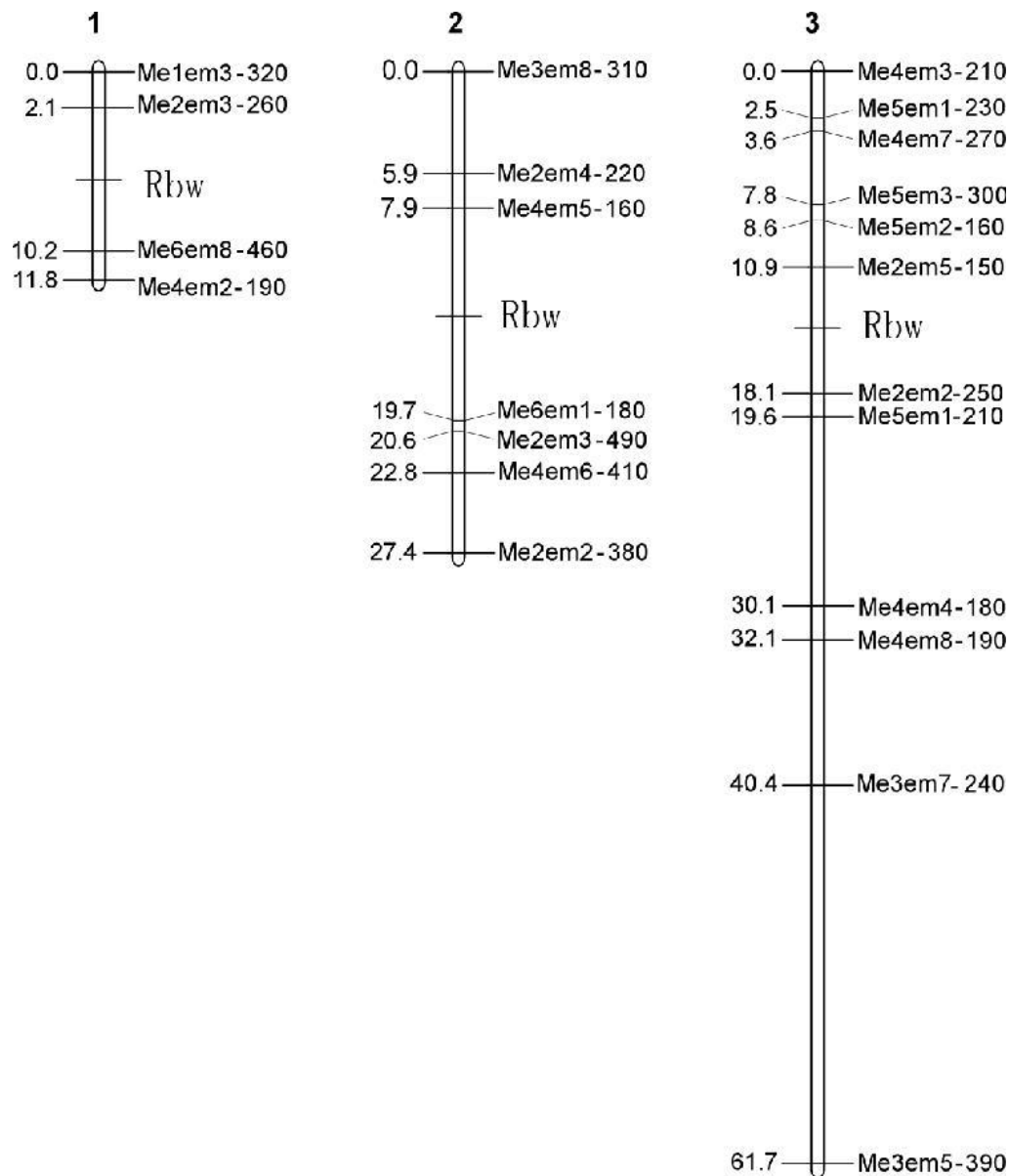


Figure 1. Genetic linkage groups of F1 population constructed with SRAP markers using the JoinMap software. Distances in centiMorgans are indicated to the left and marker names to the right followed by the fragment size in nucleotide bases. Me2em5 and Me2em2 flanked the resistance gene locus (Rbw) at genetic distances of 3.5 and 3.7 cm distance, respectively.

numbers of meioses (Zhang et al., 2012).

In this study, 16.8% of the total loci showed segregation distortion ($P < 0.05$), which is larger than other reports on cotton, wheat and *Aegilops tauschii* (Faris et al., 1998; Lin et al., 2005; Guo et al., 2007; Kumar et al., 2007; Yu et al., 2007). Skewed segregation frequently occurs in populations derived from interspecific crosses and may be influenced by many factors, such as the differential genes controlling the reproduction processes, meiotic drive controlling unique structural features and genetic properties rendering selective advantage or

disadvantage to its respective gametes or zygotes (Lyttle, 1991; Buckler et al., 1999). Both biological factors and technical problems potentially contribute to segregation distortion. Integration of distorted segregation markers in linkage construction possibly lead to untrue distance between the adjacent markers in linkage groups (Weber et al., 2003; Lu et al., 2012). Therefore, in order to increase accuracy of the genetic map constructed, the distortion segregation markers were ignored in this study. It has been proven that highly skewed markers may contribute to overestimation of recombination frequency and to

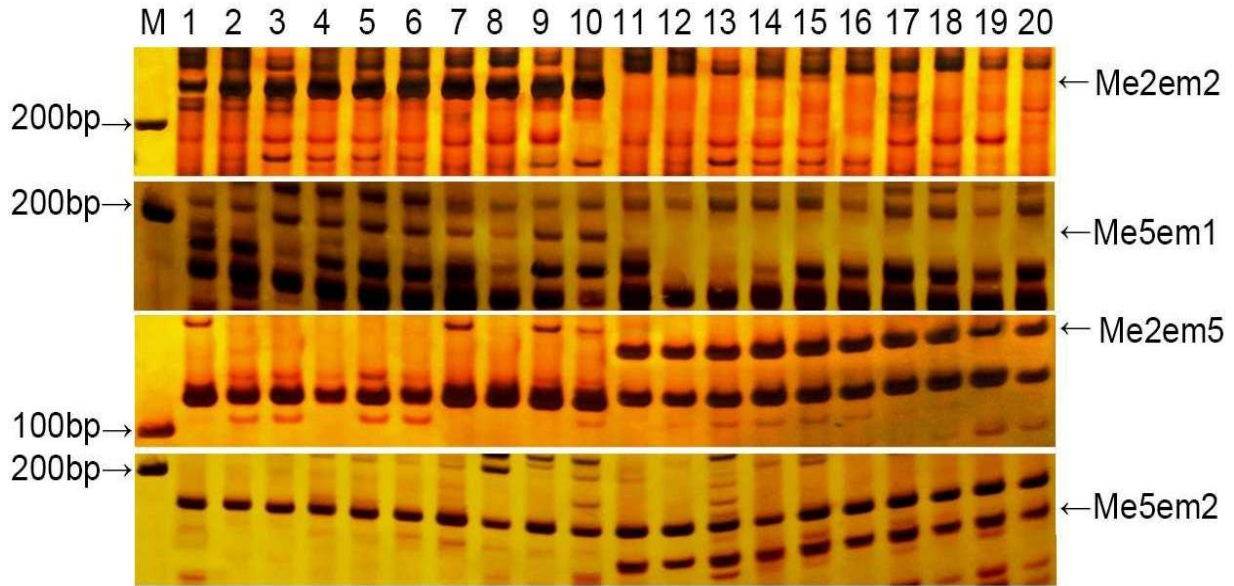


Figure 2. Samples of markers detected by primer combination Me2em2, Me5em1, Me2em5 and Me5em2 in the resistant and susceptible DNA bulks. Lanes: 1 to 10, most resistant (disease index 0) genotypes. 11 to 20, most susceptible (disease index 5) genotypes. Standard size markers are given on left side.

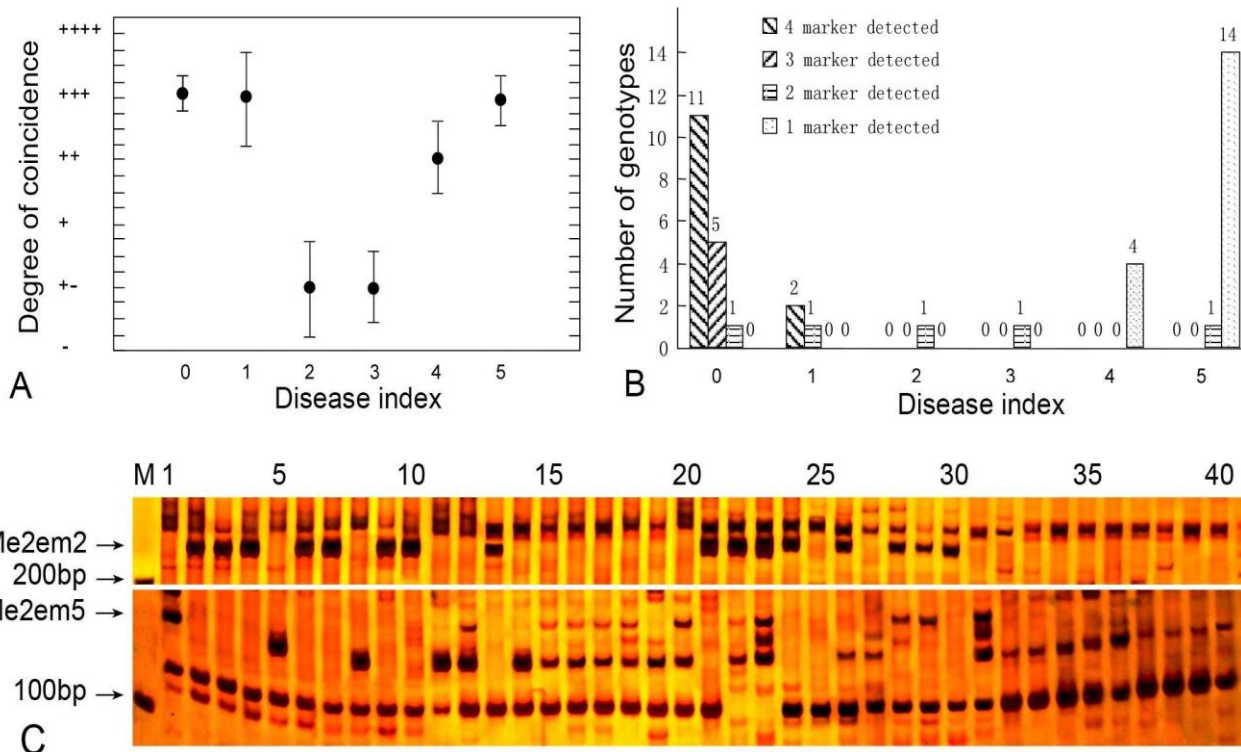


Figure 3. Assessment of the utility of 4 markers in a segregating BC1 population. A) Spearman correlation coefficient analysis for degree of coincidence between the molecular markers and the disease parameters. For disease index, 0 = no disease, 1 = 1 to 10% of leaves wilted, 2 = 10 to 25% of leaves wilted, 3 = 25 to 50% of leaves wilted, 4 = 50 to 75% of leaves wilted, and 5 = 75 to 100% of leaves wilted, or plant death 21 dpi; B) Distribution of markers genotype data from bacterial wilt resistant polymorphism and phenotype data from potato BC1 population derived from the bacterial wilt-susceptible CE26 genotype with the recurrent parent 772102.37. The results of one of three independent experiments were shown; C) Electrophoresis patterns of polymerase chain reaction-amplified with genomic DNA of 20 genotypes. M, molecular marker showed by arrows followed by base number; Lanes 1 to 10, F1 plants; lanes 11 to 20, susceptible F1 plants. Primer combinations are given on left side.

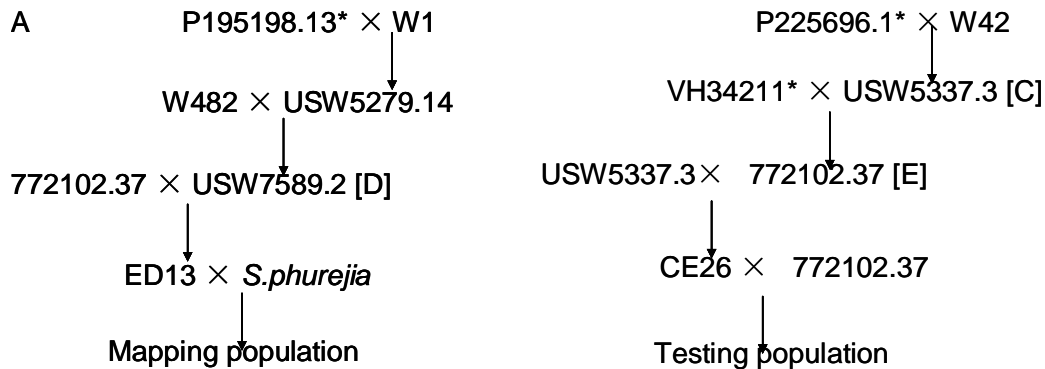


Figure 4. Genetic background of the plant materials used in this study. A) The mapping population composed of 230 genotypes; B) The testing population composed of 47 genotypes. *Pedigree from *S. phurejia* and *S. vernei* involved (Qu, 1996).

loose linkages between markers while they may cause the merging of two linkage groups (Saliba-Colombani et al., 2000). Thus, in this study, the skewed markers had to be excluded from the mapping analysis.

Five markers (5.8%) did not show strong linkage to our aim, and they were excluded from the analysis. Conversely, they may cause the merging of two linkage groups. Although, this phenomenon was manifested in others (Levi et al., 2006; Saliba-Colombani et al., 2000) and our results, we speculate that these molecular markers in group 1 or 2 may represent additional resistance genes; they are more likely to link within other different chromosomes. There may be of different origin derived from those remoter ancestors. This result suggested that the bacterial wilt resistance may be controlled by quantitative trait loci, or there may be more loci exist in potato genome. A recent report on resistance to *R. solanacearum* in eggplant (Lebeau et al., 2013) seems to be able to support this speculation. It is worthy that a more in-depth genetic analysis of bacterial wilt resistance in potato, especially in tetraploid potato, needs to be considered. The markers Me2em2, Me5em1, Me2em5 and Me5em2 found associated with the locus can be used readily for marker assisted selection, helping to introgress the recessive resistance allele of this gene into cultivated lines. Although, these are not codominant markers, the linkage in coupling phase of Me2em2, Me5em1 and in repulsion phase of marker Me2em5 and Me5em2 to the resistance allele, makes it possible to identify almost all *R. solanacearum* isolates.

Use of these markers might circumvent in many cases progeny testing of resistant plants, thereby reducing in half the time required to develop bacterial wilt resistant lines. Since the BC1 plants generated did not contain the same level of resistance found in the mapping population, as the smaller population was more likely to exhibit a larger segregation distortion and different genetic recombination; it was possible that a certain deviation of the matching degree occurred between the symptomatic phe-

notypic identification and molecular marker detection. It was not surprising that two moderately susceptible genotypes were detected to contain a molecular marker. Similar results were confirmed in others of similar experiments in eggplant (Lebeau et al., 2013). Using spearman correlation for analysis we found that there was not very strong correlation existed between the SRAP markers (4 primer recombination) and bacterial wilt disease scores. On the interpretation of the results, we inferred that the symptom and disease scores were not significantly correlated with the numbers of the SRAP markers used here. In general, the correlation between symptom scores and gene functional status measures should be stronger than the correlation between disease scores and gene number measures. After all, the other markers in linkage groups 1 and 2 were not used for correlation analysis. It is worth paying more attentions to that those markers should become the object of special consideration in MAS breeding program because the contribution from the gene loci linked with the markers is not inconsiderable.

It has been demonstrated that SRAP markers have good coverage of the genome and was able to rapidly detect markers linked to the resistance gene (Guo et al., 2012; Lu et al., 2012; Zhao et al., 2012). The results in this study suggest that the resistance to bacterial wilt is not simply inherited, but possibly controlled by a series of genes. Here, we identified the SRAP markers that proved useful in selecting for the gene conferring bacterial wilt resistance in a BC1 potato population. The set of markers provide a robust marker combination for use in MAS breeding program to identify genotypes containing the relative allele conferring bacterial wilt resistance in potato cultivars.

Conclusion

In this study, based on an F1 segregating generation, we identified the SRAP markers that proved useful in selecting for the gene conferring bacterial wilt resistance

in a BC1 potato population. The set of markers provide a robust marker combination for use in MAS breeding program to identify genotypes containing the relative allele conferring bacterial wilt resistance in potato cultivars.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Allele frequency of hyperkalemic periodic paralysis (HYPP) in quarter horses from Mexico

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Hyperkalemic periodic paralysis (HYPP) is an autosomal co-dominant genetic disease of Quarter-mile horses which originated by a point mutation of the gene coding the sodium channel protein in the plasmatic membrane of muscular cells. The mutation affects both dominant homozygous and heterozygous animals with myotonia, unpredictable muscular paralysis, weakness and collapse. In some cases, death can occur due to paralysis of the hearth or respiratory muscles. Detection of affected animals can be achieved by a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) test. Based on the fact that the mutation originated in the stallion "Impressive", whose genetic material is known to have been used in Mexico, it is possible that HYPP have been disseminated among Mexican Quarter horses. Blood samples were obtained by random sampling from 51 Quarter horses and subjected to PCR-RFLP analysis. The results obtained showed 43 recessive homozygous (N/N, normal, 84.3%), seven heterozygous (N/H, affected, 13.7%) and one dominant homozygous (H/H, affected, 2%). Allelic frequencies found were $N = 0.157$ and $n = 0.843$. The total of 15.7% affected animals can be considered a relatively high frequency of the disease; therefore, molecular diagnosis of HYPP is recommended to prevent a further spread of the mutation among Mexican Quarter horses.

Key words: HYPP, quarter horse, PCR-RFLP, Mexico.

INTRODUCTION

Hyperkalemic periodic paralysis (HYPP) is a genetic disease with an autosomal co-dominant way of inheritance which is present in quarter-mile horses and their crosses (Naylor et al., 1999). The disease has also been reported in horses of the Appaloosa and Pinto breeds (Church 1995; Rudolph et al., 1992a). It causes muscle tremors, weakness, and paralysis of respiratory

muscles, collapse and even death in some cases as a result of stress or during general anesthesia (Pang et al., 2011). Based on the fact that HYPP is a musculoskeletal disease, collapse occurs without loss of consciousness (Lyle and Keen, 2010). These symptoms appear in affected animals (dominant homozygous and heterozygous) between two and three years old.

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Table 1. Number of animals analyzed by location.

State sampled	County of animals	Number of animals sampled
Nuevo León	Allende	13
	Santiago	13
Coahuila	Arteaga	8
	Saltillo	10
Tamaulipas	Villagrán	7
Total		51

Symptoms are more severe in the dominant homozygous than in the heterozygous animals, and can be managed successfully given a rapid diagnosis and treatment (Naylor et al., 1999; Pang et al., 2011). Signs of the disease are manifested by a sudden increase in the blood serum potassium concentration, above 8 to 9 mEq/L, indicating an alteration of the normal potassium entrance and exit from muscle cells (Naylor 1994; Zeilmann et al., 1993). The disease is caused by a point mutation originating from the substitution of a cytosine for a guanine and the concomitant replacement of a leucine for a phenylalanine in the gene coding the sodium channel protein (transmembranal S region, subunit alpha) of muscle cells (Cannon et al., 1995; Rojas et al., 1991; Rudolph et al., 1992b; Valbers, 2010).

HYPP was originated with the stallion "impressive", which had a wide diffusion as a breeder in the U.S.A (Bowling et al., 1996; Naylor et al., 1994; Rudolph et al., 1992a). Reported frequencies of HYPP include studies in the U.S.A., in which a frequency of 4.4% of animals (frequency of 0.044) with the mutation was reported for Quarter Horses, corresponding to a allelic frequency of 0.02 (Bowling et al., 1996), as well as frequencies ranging from 0.008 in Quarter Horses to 0.299 in halter American paint horses (Tryon et al., 2009). The mutation has not been found nor described in other breeds or subtypes of horses and no further information of its frequency in horses exist. It is suspected that its lineage has extended into Mexico, since animals belonging to the "Impressive" genetic line do exist in this country. However, in order to determine the frequency of HYPP, random testing (not including only a single blood line) is needed (Bowling et al., 1996). Presently, no such studies exist in México.

The objective of the present work was to establish the genotype and allelic frequencies of HYPP by PCR-RFLP in an open population of Quarter horses and its crosses located at northern Mexico. These results will have an impact on further spreading the disease, since they could lead to the implementation of a screening program to identify animals with the mutation, assuring the genetic quality of the animals.

MATERIALS AND METHODS

Blood samples were taken from 51 Quarter-mile horses chosen at random from three states and five counties located in north-east Mexico (Table 1). In this procedure, horses were sampled with no knowledge of their pedigree; therefore, the sampled horses did not belonged exclusively to animals with the "Impressive" blood line, but to the general Mexican Quarter-mile horse population. Molecular diagnosis of HYPP allows the determination of genotypes for the disease with a 99% precision (Bowling et al., 1996; Duyn and Hering, 1995). It consists of the amplification by polymerase chain reaction (PCR) of a 92 base pair (bp) segment of the gene coding the cellular sodium channel followed by its digestion with the restriction enzyme *Taq* I (restriction fragment length polymorphism, RFLP). *Taq* I cuts the PCR product when the mutation is absent, originating into two fragments (64 and 28 bp). The mutation destroys the *Taq* I restriction site and therefore no digestion occurs when it is present (Rudolph et al., 1992b).

The Puregene kit (genomic DNA Purification kit, Gentra Systems) was used for DNA extraction. PCR primers used were: IVS₂F4: 5'-GGGGAGTGTGTGCTCAAGATG-3'; IVS₃R: 5'-AATGGACAGGATGACAACCAC-3'. These primers amplify a 92 base pair (bp) DNA fragment containing the mutation. PCR conditions were as follows: initial denaturalization at 94°C for 5 min; 35 cycles of denaturalization at 94°C for 1 min, hybridization at 60°C for 1 min, extension at 72°C for 1 min; a final extension at 72°C for 10 min. To each sample of 5 µL genomic DNA diluted to 20 ng/µL was added a reaction mix composed of 10 µL of *Taq* and Go PCR mix (Gentra Systems) containing *Taq* DNA polymerase (2.5 U/sample), PCR buffer solution (1x), dNTP's (200 µM) and MgCl (1.5 mM), plus 0.5 µL each PCR primer (20 pM) and 34 µL molecular grade water for a final volume of 50 µL. Amplifications were carried out in a hot-lid thermocycler, always including a negative control (without DNA). An aliquot of PCR products obtained were subjected to digestion with restriction enzyme *Taq* I for 1 h at 65°C (Rudolph et al., 1992b).

Both the PCR and digestion products were visualized by 30% polyacrylamide gel electrophoresis, staining the DNA with ethidium bromide for detection with a photo documenter according to standard procedures. In order to determine genotypes, fragment size was determined by comparison with molecular ladders. Genotypes: dominant homozygous, H/H, affected: one 92 base pair (bp) band; heterozygous, N/H, affected: one 92, one 64 and one 28 bp band; recessive homozygous, N/N, unaffected: one 64 and one 28 bp band. Genotype frequencies were determined by direct count of animals according to the molecular diagnostic, dividing the number of animal with each genotype between the total numbers of analyzed animals. Allelic frequencies were calculated by direct gene counting, following the standard formulae showed below:

N allele: frequency of $\frac{N/H}{2}$ + frequency of N/N; H allele: 1 - N allele.

RESULTS

The undigested PCR product (92 bp) was obtained from all 51 samples (Figure 1). RFLP analysis showed that the expected 28 bp band could not be detected; however, it was possible to determine the genotype of the animals based on the presence of the other expected bands, since the N/N genotype had only one 64 bp band, the N/H had a 92 and a 64 bp bands and the H/H had only one 92 bp band (Figure 2). Based on this, one animal was dominant homozygous (frequency of 0.02), 7 were heterozygous (0.14) and 43 animals were recessive

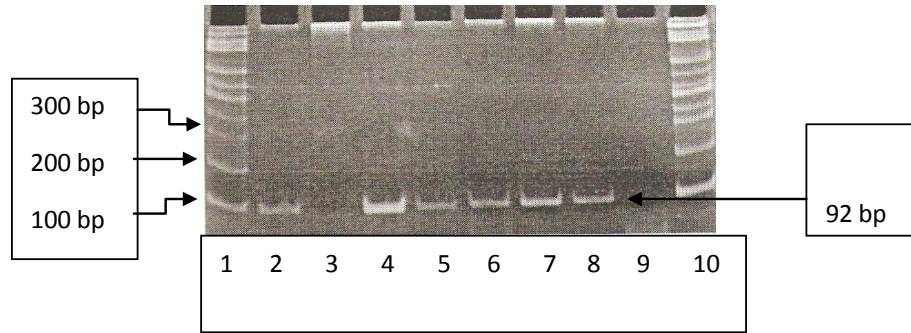


Figure 1. Visualization of PCR amplification of the HYPP gene. The expected PCR product of 92 base pairs (bp) is shown (lanes 2 and 4 - 8). Lane 3 shows no amplification in this particular experiment and lane 9 is the negative control. Lanes 1 and 10: molecular size marker (100 bp ladder).

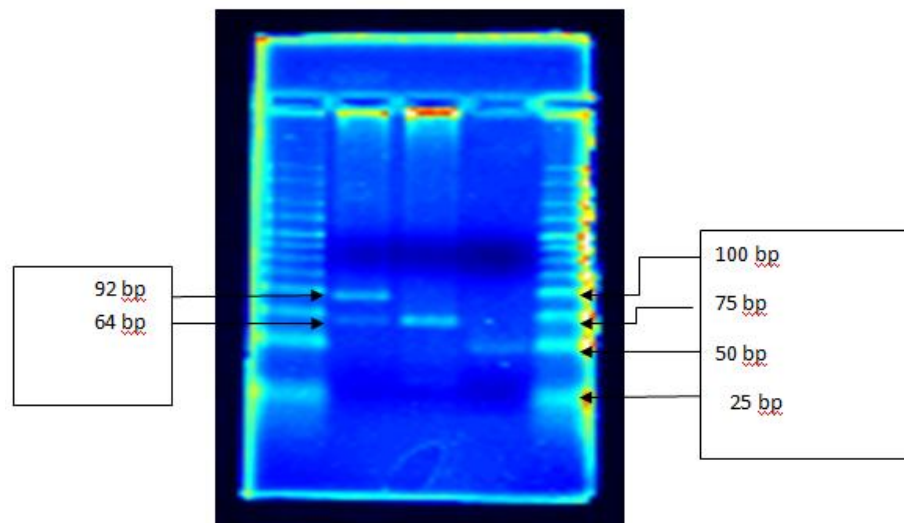


Figure 2. Visualization of PCR-RFLP analysis. Lane 2 shows the presence of the 92 and 64 base pair (bp) size bands (expected in genotype Nn). Lane 3 contains only the 64 bp size band (expected in genotype nn). The expected 28 bp band is missing in both lanes 2 and 3; however, both the heterozygous affected (Nn) and the normal (nn) genotypes can be distinguished from the homozygous affected (NN) genotype (only a 92 bp band, result not shown). The close to 50 base pair size band in lane 4 is a primer dimer and contained a negative control. Lanes 1 and 5: molecular weight marker (25 b.p. ladder).

homozygous (0.84). In total, 8 animals (0.157) presented the mutation. Allelic frequencies were H = 0.157 and N = 0.843 (Table 2). The 28 bp band was not detected due to its small size, which makes it difficult to visualize in gels.

DISCUSSION

The frequency of animals with the mutation in the studied population (0.157) can be considered moderately high when compared to previously informed frequencies ranging from 0.008 to 0.299 in the U.S.A. from Quarter Mile horses sampled at random (Bowling et al., 1996; Tyron et

al., 1996). In the present paper, evidence exists of the introduction of HYPP into the Netherlands (Sloet van Oldruitenborgh-Oosterbaan, 1999) and Australia (Church, 1995); furthermore, current cases of HYPP in quarter horses, confirmed by DNA analysis, do exist in countries such as Canada (Pang et al., 2011). These results are in agreement with those presented in this paper and confirm the need of strengthening the procedures for the verification of the status for HYPP by laboratory analysis. Some evidence at the Western part of México exists on the presence and frequency of animals with HYPP. At a local Congress held in 1995, a frequency of animals with HYPP of 40% (0.4) was found among animals that shared

Table 2. Number and frequency of animals by genotype.

Genotype (%)	Number of animals	Genotype frequency
Dominant homozygous (NN, affected)	1	1.96 (1/51)
Heterozygous (Nn, affected)	7	13.70 (7/51)
Recessive homozygous (nn, normal)	43	84.30 (43/51)

the “Impressive” blood line (Ayala-Valdovinos et al., 2006, unpublished results).

As mentioned earlier, random sampling is needed in order to obtain the allelic frequency. The frequency of animals found in the present study (0.157) is considerably lower than the mentioned Western México, which can be explained for the kind of animals sampled. However, our results give additional evidence of relatively high allelic frequency for HYPP in quarter horses in Mexico. In our study, close to 16% of the animals sampled were positive for HYPP. We considered that the results obtained in the present study are only applicable to the Mexican north-east Quarter-mile horse population, and in order to determine the whole frequency of horse HYPP in Mexico, further studies should be performed at other areas of the country, as well as in other horse populations such as in the Pinto and Appaloosa breeds.

This result indicates that although the pedigree record must include a certification of HYPP-free of Quarter horses imported from the U.S.A. to Mexico, the mutation still exists in the genetic pool of this breed and its crosses. However, since no such laboratory test is required for animals born inside Mexico, a genetic test based on PCR-RFLP analysis could contribute to the elimination of the disease from the quarter horse population in this country, which is in agreement with the conclusions obtained by other authors indicating that the use of DNA analysis for selective breeding is important for the reduction and eradication of HYPP, and that only normal (N/N) horses should be bred (Nollet and Deprez, 2005).

On the other hand, the American Quarter Horse Association have ruled that foals born in 2007 or later that tested homozygous for HYPP (H/H) would not be eligible for registration (Bettley et al., 2012). This type of measures could be implemented in Mexico in order to obtain the same goal of reducing or eradicating HYPP.

Conflict of interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Transcriptional profiling of three key genes of terpenoid indole alkaloid pathway in *Catharanthus roseus* under different tissue culture conditions

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The response of three key genes: strictosidine synthase (*str1*), tryptophan decarboxylase (*tdc*) and secologanin synthase (*cyp72A1*) of the wild plant species, *Catharanthus roseus* to different plant tissue culture treatments was studied. These genes encode enzymes acting early in the biosynthetic pathway of terpenoid indole alkaloids. *In vitro* culture system conditions involved the application of three sucrose (40, 50 and 60 g/L), three benzyl adenine (0.1, 0.2 and 0.4 mg/L) and two jasmonic acid (10 and 100 µM) concentrations. Quantitative RT-PCR (qRT-PCR) using SYBR Green I was used to analyze the changes in expression of the three genes in response to different media recipes. The maximum folding of *str1* expression (1.9x) between treated and untreated callus was obtained under ba2 (0.2 mg/L benzyl adenine) treatment. Relatively high folding values of 1.8x and 1.7x were obtained in S2 (50 g/L sucrose) and ba1 (0.1 mg/L benzyl adenine), respectively. Two-fold increase in gene expression of *tdc* was obtained when *C. roseus* callus was treated with 10 µM jasmonic acid (ja1), while only 1.5x was obtained when callus was treated with 100 µM jasmonic acid (Ja2). The maximum expression of *cyp72A1* gene (4.6x) was observed under ba2 treatment, when the callus was treated with 0.2 mg/L benzyl adenine. This emphasized the influence of BA on up-regulation of this gene.

Key words: Strictosidine synthase, tryptophan decarboxylase, secologanin synthase, *Catharanthus roseus*, terpenoid indole alkaloids.

INTRODUCTION

Catharanthus roseus (L.) is one of the most extensively investigated medicinal plants and is known mainly for its pharmacologically important alkaloids (Verpoorte et al.,

1997). At present, the *Catharanthus* alkaloids comprise a group of about 130 terpenoid indole alkaloids (TIAs) (van der Heijden et al., 2004). The plant is particularly known

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for its economically important leaf-specific bisindole alkaloids, vinblastine and vincristine, which are potent anti-neoplastic agents (Svoboda and Blake, 1975) and indispensable constituents of most cancer chemotherapies. The lack of knowledge on the fully functional pathways required for the production of target molecules is pointed out as the main reason why the attempts to use plant tissue and cell cultures as alternative sources of natural products have been problematic (Zhou et al., 2010).

More than 100 *C. roseus* alkaloids that have been identified share many common biosynthetic steps. Early monoterpene indole alkaloid biosynthesis begins with the condensation of tryptamine, which is derived from the decarboxylation of tryptophan by tryptophan decarboxylase (*tdc*) with secologanin, which is derived from the iridoid pathway by secologanin synthase (*cyp72A1*) that converts loganin to secologanin (De Luca, 1993). These two moieties are enzymatically condensed by strictosidine synthase (*str1*). The strictosidine, then, serves as universal precursor of the two secondary compounds: Vinblastine and vincristine (De Luca et al., 1986).

The production of secondary metabolites by tissue culture system has become an active field of study because of its potential as a source of valuable pharmaceutical compounds. In this context, *in vitro* cultures of plant cells or tissues look promising for the large scale production of secondary metabolites (Tikhomiroff and Jolicoeur, 2002; Mulabagal and Tsay, 2004).

Real-time PCR, which combines the advantages of conventional PCR with quantitative capability (Simpson et al., 2000) is one of the most sensitive and accurate methods for the detection and quantification of gene expression (Giulietti et al., 2001). Its high sensitivity allows the quantification of rare transcripts and small changes in gene expression (Schmittgen and Zakrajsek, 2000; Rajeevan et al., 2001; Vandesompele et al., 2002; Kim et al., 2003; Radonic et al., 2004).

This study aimed at showing the response of three key genes: Strictosidine synthase (*str1*), tryptophan decarboxylase (*tdc*) and secologanin synthase (*cyp72A1*) that act early in the biosynthetic pathway leading to terpenoid indole alkaloids, to different tissue culture treatments.

MATERIALS AND METHODS

Seeds of red purple variety of Egyptian *C. roseus* were kindly obtained from Institute of Horticulture Research, Agricultural Research Centre, Giza, Egypt.

Preparation of plant material, media and culture conditions

Seeds were surface sterilized under aseptic conditions of laminar flow hood, using 12% H₂O₂ for 5 min. Then they were aseptically germinated on half-strength solid basal Murashige and Skoog (MS) medium including micro and macro elements and vitamins (Murashige and Skoog, 1962) in tissue culture incubator. After germination, the seedlings were transferred to growth chamber in

pots containing soil at 25 ± 3°C. Leaves were excised from 4 to 6 weeks old seedlings and used as a source of explant materials for calli production.

Callus initiation and treatments

Callus cultures were initiated from fresh *C. roseus* leaf fragments. Transversal leaf sections (2 mm) were transferred to jars containing 30 to 40 ml of solid medium. MS medium supplemented with sucrose at a concentration of 30 and 1 g/L of 2,4 dichlorophenoxy acid (D) + 0.1 mg/L of kinetin (Kin) was routinely used for *C. roseus* callus induction and growth and as a control medium in the different treatment experiments. Cultures were incubated at 16 h light and 8 h dark and 22 to 25°C. All culture media used in this study were adjusted to pH = 5.6 to 5.8 before solidification with 0.2% GELRITE.

After four weeks from callus initiation, the callus was weighted according to the study of Zhao et al. (2001) and sub-cultured to the treatment media. In this study, three different sucrose concentrations (40, 50 and 60 g/L) and three concentrations of benzyl adenine (0.1, 0.2 and 0.4 mg/L) in addition to two concentrations of jasmonic acid (10 and 100 µM) were applied to determine their influence on the expression of three key genes of TIAs pathway in *C. roseus* tissue culture.

Primer design for real time PCR analysis

Both forward and reverse primers, for recovering the full-length of the three *C. roseus* genes, were designed based on the known nucleotide sequences obtained from the GenBank database. These genes are strictosidine synthase (*str1*) (GenBank Acc. No. X61932), tryptophan decarboxylase (*tdc*) (GenBank Acc. No. X67662) and secologanin synthase (*cyp72A1*) genes (GenBank Acc. No. L10081). In designing these primers, one of the most important criteria to take into account is the targeting of relatively small amplicon size of not more than 250 bp. DNASTAR V. 7.0.0 software and Primer-3 V.4 software were used to design the primers. The designed primers were synthesized by Metabion, Germany. Primer pairs of candidate endogenous reference gene *CrActin* (no accession number available) were designed according to the study of Jiao and Deng (2007). Primer sequences of the three target as well as the endogenous gene used for real-time PCR and their annealing temperatures are shown in Table 1.

Real time quantitative RT-PCR

Total RNA was extracted by TRI Reagent[®] RNA isolation (Sigma T9424) as describe in the manufacturer's manual. RevertAid[™] H⁻ M-MuLV reverse transcriptase (thermo scientific, Fermentas, Lithuania) was used in the reverse transcription and real time PCR. Total RNA from both treated and untreated *C. roseus* calli were DNase-treated prior to cDNA synthesis. Five hundred nanograms of RNA from each of the treatments were reverse transcribed according to the manufacturer's protocol. The 20 µl reaction consisted of 2x RT reaction mix (2.5 µM Oligo(dT)18, 10 mM MgCl₂ and 0.2 uM dNTP), 2 µl RT Enzyme Mix (RevertAid[™] H⁻ M-MuLV RT and RNaseOUT) and DEPC-treated water. All the components were mixed and incubated at 25°C for 10 min. Further incubation at 42°C for 50 min was carried out. The reaction was terminated by incubating at 85°C for 5 min and then chilled on ice.

Real-time PCR was carried out using the Agilent Mx3000P QPCR systems (Agilent technology, USA). All cDNA samples synthesized were diluted 1:10 prior to amplification. Each reaction was performed in a 25 µl volume. The reaction components were 12.5 µl Maxima[™] SYBR Green/ROX qPCR Master Mix, 0.2 µM of

Table 1. Primer sequences of the three target genes and the endogenous gene used for real-time PCR.

Gene ID	Primer sequences	Annealing temperature (°C)
<i>tdc</i>	F 5'-TTC TTC ACC AGC CGC CAC CG-3' R 5'-GCG CTT CCC GCA TAA GCA GC-3'	62
<i>str1</i>	F 5'-TGA GGC CAC CTA CCA TCC CGT-3' R 5'-GCA GCA GAC ACT CAA AAT CTC CTC C-3'	62
<i>CYP72A1</i>	F 5'-ACC GGA GTT GGA AGC TTT GAG GGT-3' R 5'-TCC TGC AGG GAT TGT GTA CGA CC -3'	64
<i>CrActin</i>	F 5'-GGC TGG ATT TGC TGG AGA TGA T -3' R 5'-TAG ATC CTC CGA TCC AGA CAC TG -3'	57.6

each forward and reverse primer, and PCR-grade water to make up the volume to 22.5 µl. Finally, 2.5 µl of diluted cDNA template was added to the reaction mix. Amplification for each sample was carried out in triplicate along with a no-template control (NTC) in which PCR-grade water was used as template. The thermal cycling conditions consisted of 1 cycle at 95°C with a 2 min hold for denaturation (hot-start) and followed by 40 cycles of denaturation at 95°C for 15 s, annealing as shown in Table 1 for 30 s and extension at 72°C for 30 s. Data were collected and amplification plots of ΔR_n versus cycle number were generated for analysis. To confirm that only one PCR product of the expected size was amplified in the treated and untreated samples, all the reaction products were further analyzed by agarose gel electrophoresis as described by Liu et al. (2011).

Data analysis

The data were analyzed and amplification plots were generated using the software program, MxPro QPCR software which is part of the Agilent Mx3000P QPCR systems. The standard curves were generated using both the software programs, and Microsoft Excel. The $\Delta\Delta C_T$ calculation for the relative quantification of target gene was used as follows $\Delta\Delta C_T = (C_{T, \text{target gene}} - C_{T, \text{CrActin}})_{\chi} - (C_{T, \text{target gene}} - C_{T, \text{CrActin}})_{y}$, where χ = treated sample and y = control sample. After validation of the method, results for each sample were expressed in N-fold changes in χ target gene copies, normalized to *CrActin* relative to the copy number of the target gene in control, according to the following equation: amount of target = $2^{-\Delta\Delta C_T}$ (Livak and Schmittgen, 2001).

RESULTS AND DISCUSSION

Researchers aim to produce substances with antitumor, antiviral, hypoglycaemic, anti-inflammatory and antimicrobials properties through tissue culture technology. However, establishment of tissue culture system is required prior to further exploration of the biosynthetic capabilities of various cell cultures. Tissues from various organs such as stem and leaf of the axenic plantlets can be induced to form callus. Callus tissue can serve as an experimental system to investigate the biological activities using specific bioassays. However, many factors contribute to the ability of a specific tissue to form callus such as

medium and plant growth regulators.

Establishment of tissue culture system for Egyptian *C. roseus*

The red purple variety of Egyptian *C. roseus* was used in the present study as a source of explants for callus initiation. In a preliminary experiment, three explant types were used: hypocotyl (undeveloped lower stem), cotyledons and mature leaves. Transversal leaf sections proved to be the best explant, which resulted in production of healthy callus with good size, shape and color on MS medium (data not shown).

Effect of different sucrose concentrations on *C. roseus* callus growth

Increased sucrose concentration usually results in increased biomass and secondary metabolite production of plant cell cultures (Zhao et al., 2001). Five different sucrose concentrations were tested in the present study for their effect on callus growth. In response to increased sucrose concentrations (from 20 to 60 g/l) calli changed most of their characteristics such as color, size and the degree of compaction (decreased ratio of FW/DW). As shown in Table 2 and Figure 1, the ratio of fresh weight (FW) to dry weight (DW) of the calli varied from 3 to 25.24. The ratio of FW/DW can reasonably represent the degree of compaction of the callus structure. More organized and compacted callus usually gave lower ratios of FW/DW (Zhao et al., 2001).

Effect of phytohormone balance on *C. roseus* callus growth

Plant hormones, like animal hormones, are relatively small molecules that are effective at low tissue concentrations. The two types of plant hormones used in

Table 2. Effect of sucrose concentrations on *C. roseus* callus characteristics

Sucrose (g/L)	Callus characteristic				
	Color	Texture	(FW) mg*	(DW) mg*	FW/DW
20	Off white	Loose	85.8	3.4	25.24
30	Off white	Loose	19.94	1.61	12.39
40	dark yellow	Compact	23.02	2.18	10.56
50	dark yellow	Compact	23.4	2.2	10.63
60	Greenish yellow	Hard	18.6	6.2	3

*Mean of three calli, FW = fresh weight, DW = dry weight.

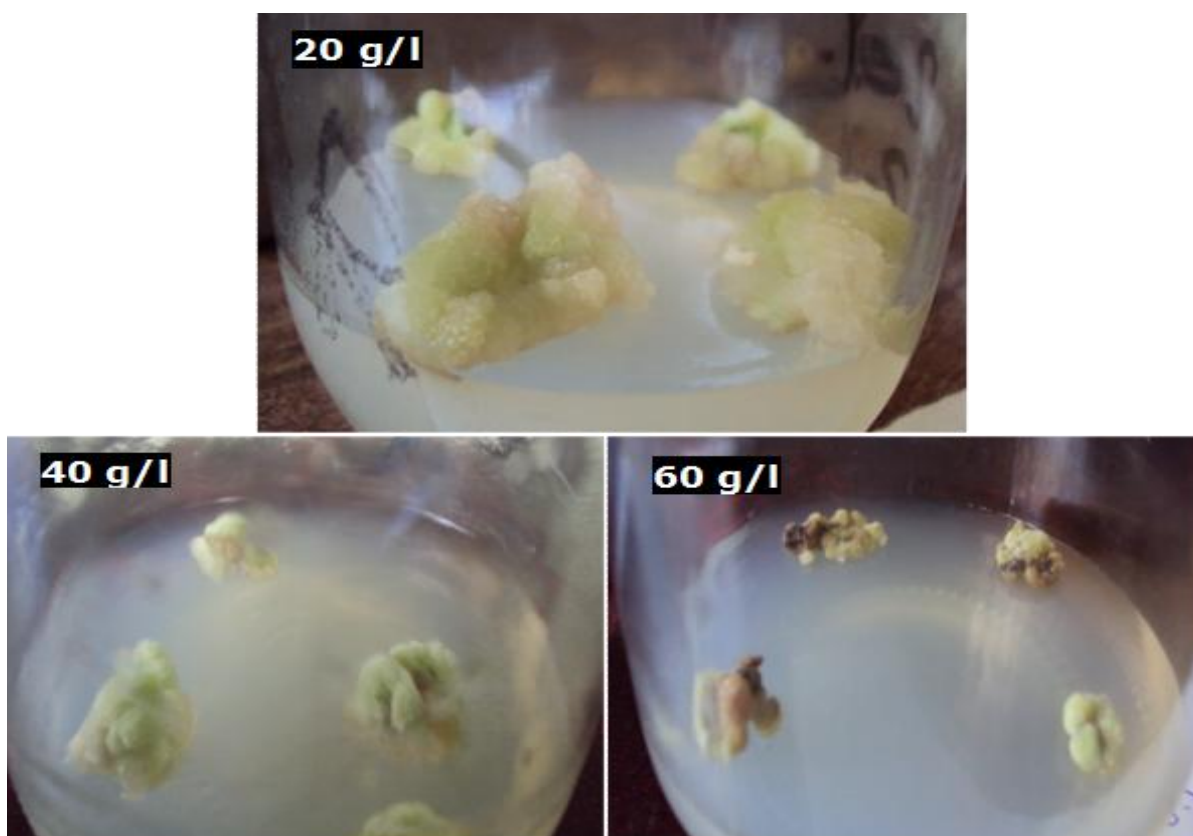


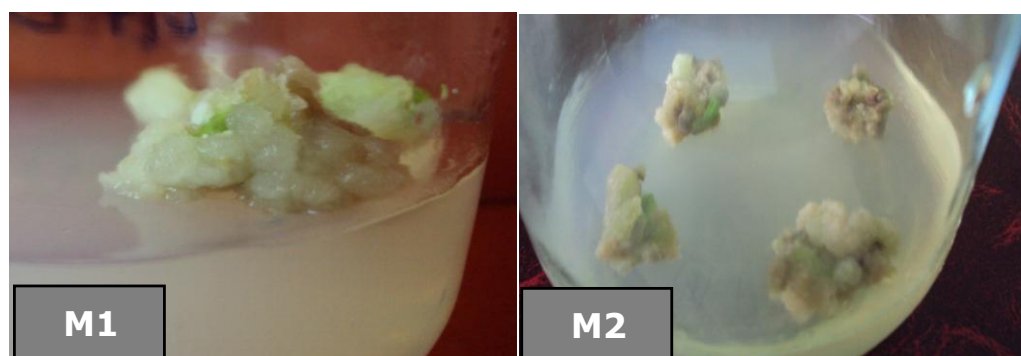
Figure 1. Effect of different sucrose concentrations on color, size and the degree of compaction of *C. roseus* callus.

this experiment are cytokinins and auxins. Cytokinins are derived from adenine and produce two immediate effects on undifferentiated cells: the stimulation of DNA synthesis and increase cell division (Ting, 1982). Auxins are indole or indole-like compounds that stimulate cell expansion, particularly cell elongation. Auxins also promote adventitious root development. Plant hormones do not function in isolation within the plant body, but, instead, function in relation to each other. Hormone balance is apparently more important than the absolute concentration of any one hormone. Both cell division and cell expansion occur in actively dividing tissue, therefore cytokinin and auxin balance plays a role in the overall growth of plant tissue

(Xing et al., 2011). Four plant hormones were used in this study, two auxins (2,4 D and NAA) and two cytokinins (kinetin (Kin) and benzyl adenine (BA)). Four combinations of these plant hormones (M1 to M4) were tested for their effects on callus induction and growth. Qualitative and quantitative differences were observed and the results are shown in Table 3 and Figure 2. Based on morphological appearance, different types of calli were observed. Among the different combination, best results were obtained on the medium supplemented with 1 mg/L 2,4D + 0.1 mg/L Kin (M1) after four weeks of callus initiation. It was also observed that replacing 2,4 D with NAA gave the same result, but the callus took about

Table 3. Effect of four media containing different combinations of plant growth regulators on *C. roseus* callus characteristics.

Media	Growth regulator				Callus characteristic		
	Auxin (mg/L)		Cytokinin (mg/L)		Color	Texture	Texture
	2,4D	NAA	Kin	BA			
M1	1	0	0.1	0	Off white	Friable	Friable
M2	1	0	0	0.1	Dark yellow	Compact	Compact
M3	0	1	0.1	0	Dark yellow	Compact	Compact
M4	0	1	0	0.1	Dark yellow	Compact	Compact

**Figure 2.** Effect of two combinations: M1 (1mg/l 2,4D + 0.1 mg/L kin) and M2 (1 mg/l 2,4D + 0.1 mg/L BA) of plant growth regulators on callus characteristics.

6 weeks to reach the desired size and shape.

Callus culture treatments

The significant advances in plant tissue culture techniques have led to the use of callus and cell suspension culture (undifferentiated cells) of some plant species for the study of biological activities and production of valuable secondary metabolites (Mulabagal and Tsay, 2004). In efforts to improve the production of alkaloids, cell cultures of *C. roseus* received considerable attention. The terpenoid indole alkaloids pathways are not only regulated tissue specifically and developmentally (Facchini, 2001), but also affected by external factors (Facchini, 2001; Zhao et al., 2001; Xing et al., 2011).

These factors include media components, phytohormones (growth regulators), pH, temperature, aeration, agitation, light, etc. (Moreno et al., 1995; Xing et al., 2011). Based on the results of the previous experiments, MS medium supplemented with sucrose at a concentration of 30 g/L and 1 mg/L of 2,4D + 0.1 mg/L of kin proved to be more appropriate for callus induction and growth of the Egyptian *C. roseus* and routinely used in this study for callus production and as a control medium in the different treatment experiments. Three sucrose concentrations (S1 = 40, S2 = 50 and S3 = 60 g/L) and three concentrations of benzyl adenine (ba1 = 0.1, ba2 =

0.2 and ba3 = 0.4 mg/L) in addition to two concentrations of jasmonic acid (ja1 = 10 μ M and ja2 = 100 μ M) were applied to determine their influence on regulation of gene expression of three key genes in TIAs pathway in *C. roseus*. Sucrose and glucose are the preferred carbon source for plant tissue cultures. The concentration of the carbon source affects cell growth and yield of secondary metabolites in many cases. Among a number of other components in the medium phytohormones are auxins and kinetins which have shown the most remarkable effects on growth and productivity of plant metabolites (Yahia et al., 1998; Doran, 2000).

The effects of plant growth regulators on the contents of *C. roseus* TIAs had been extensively studied (El-Sayed and Verpoorte, 2007; Zhao and Verpoorte, 2007; Pan et al., 2010). Jasmonic acid (JA) proved to have significant influence on TIAs production and enzymes activities of the biosynthesis pathways in *C. roseus* cell suspension cultures, hairy roots and seedlings (El-Sayed and Verpoorte, 2005; Peebles et al., 2009).

Quantification of gene expression using real-time PCR

Real-time PCR were carried out by using primers that produced single bands (Figure 3). For amplification of each target gene from mRNA, the *CrActin* gene was also

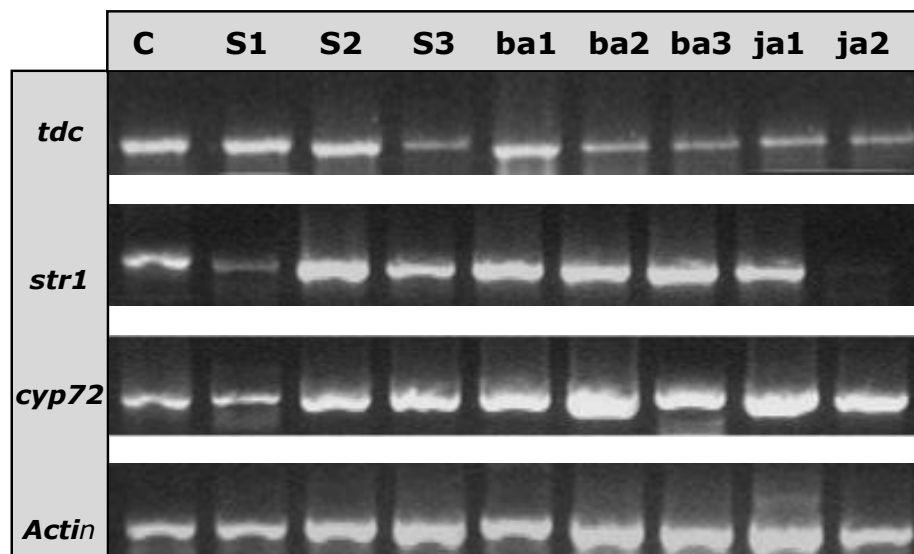


Figure 3. qPCR reaction products for the target genes (*str1*, *tdc* and *cyp72A1*) and the reference gene (*CrActin*) under treated and untreated calli. S = sucrose treatment; ba = benzyl adenine treatment; ja = jasmonic acid treatment.

Table 4. The level of *str1* gene expression between treated and untreated (control) *C. roseus* determined by the comparative $\Delta\Delta C_T$ method.

Treatment	C_T <i>str1</i>	C_T <i>actin</i>	ΔC_T	$\Delta\Delta C_T$	Folding
Control	21.54	26.24	-2.61	0	1
S1	23.92	26.14	-2.22	0.39	0.7
S2	23.82	27.29	-3.47	-0.86	1.8
S3	22.52	25.4	-2.88	-0.27	1.2
BA1	22.9	26.26	-3.36	-0.75	1.7
BA2	23.68	27.19	-3.51	-0.9	1.9
BA3	20.48	23.52	-3.04	-0.43	1.3
Ja1	24.79	27.71	-2.92	-0.31	1.2
Ja2	26.78	27.65	-0.87	1.74	0.3

included as an endogenous reference. An amplification plot was generated for each target gene and *CrActin* gene pair and C_T values were then obtained. Based on the C_T values, the ΔC_T treated and ΔC_T control values which have been normalized to the *CrActin* gene were determined. The $\Delta\Delta C_T$ value which represents the difference in transcript level between the treated and untreated callus was then calculated. The amount of transcript in the treated callus, normalized to an endogenous reference and relative to the untreated callus is expressed as $2^{-\Delta\Delta C_T}$. A positive $\Delta\Delta C_T$ value indicated down-regulation, while a negative $\Delta\Delta C_T$ value indicated an up-regulation of gene transcription (Radonic et al., 2004). The fold differences in the expression level of normalized treated callus relative to different untreated cDNA are shown in Tables 4, 5 and 6.

Transcriptional profiling of *str1* gene under different treatments

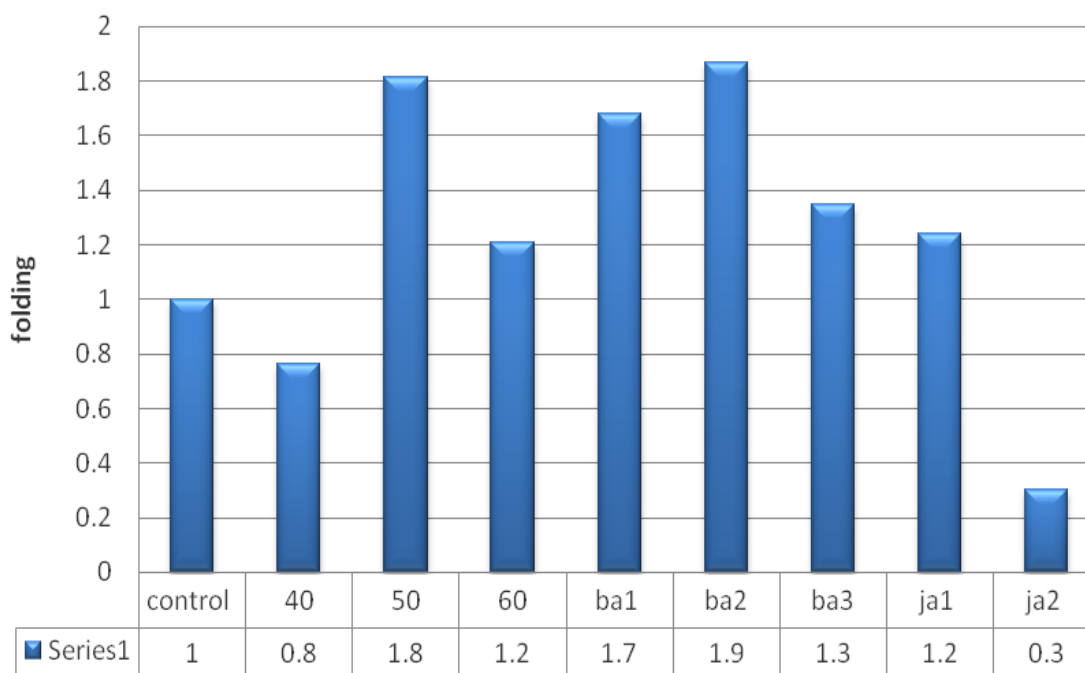
Table 4 and Figure 4 represent the folding levels of *str1* gene expression for treated and untreated (control) calli of *C. roseus* determined by the comparative $\Delta\Delta C_T$ method. Positive values of $\Delta\Delta C_T$ were detected in two cases only among the eight treatments: S1 (40 g/L sucrose) and ja2 (100 μ M jasmonic acid) indicating down-regulation of this gene in the two treatments. On the other hand, the maximum folding of *str1* expression (1.9x) between treated and untreated callus was obtained under ba2 (0.2 mg/L benzyl adenine) treatment as shown in Table 4 and Figure 4. Relatively high folding values of 1.8x and 1.7x were also observed in S2 (50 g/L sucrose) and ba1 (0.1 mg/L benzyl adenine), respectively.

Table 5. The level of *tdc* gene expression between treated and untreated (control) *C. roseus* determined by the comparative $\Delta\Delta C_T$ method.

Treatment	C_T <i>tdc</i>	C_T <i>actin</i>	ΔC_T	$\Delta\Delta C_T$	Folding
Control	23.66	26.24	-2.61	0	1
S1	24.32	26.84	-2.52	0.09	0.9
S2	19.54	22.29	-2.75	-0.14	1.1
S3	19.34	22.61	-3.27	-0.66	1.6
BA1	18.61	20.56	-1.95	0.66	0.6
BA2	20.7	23.79	-3.09	-0.48	1.4
BA3	22.4	25.52	-3.12	-0.51	1.4
Ja1	19.09	22.71	-3.62	-1.01	2.0
Ja2	22.93	26.15	-3.22	-0.61	1.5

Table 6. The level of *cyp72A1* gene expression between treated and untreated (control) *C. roseus* determined by the comparative $\Delta\Delta C_T$ method.

Treatment	C_T <i>cyp</i>	C_T <i>actin</i>	ΔC_T	$\Delta\Delta C_T$	Folding
Control	23.63	26.24	-2.61	0	1
S1	23.47	26.54	-3.07	-0.46	1.4
S2	14.64	18.29	-3.65	-1.04	2.0
S3	15.05	19.1	-4.05	-1.44	2.7
BA1	15.07	19.56	-4.49	-1.88	3.7
BA2	14.97	19.79	-4.82	-2.21	4.6
BA3	15.56	19.52	-3.96	-1.35	2.5
Ja1	15.04	19.71	-4.67	-2.06	4.1
Ja2	20.44	24.65	-4.21	-1.6	3.0

**Figure 4.** The folding levels of *str1* gene expression between treated and untreated (control) *C. roseus*. 40, 50 and 60 = sucrose treatments (g/l); ba = benzyl adenine treatment; ja = jasmonic acid treatment.

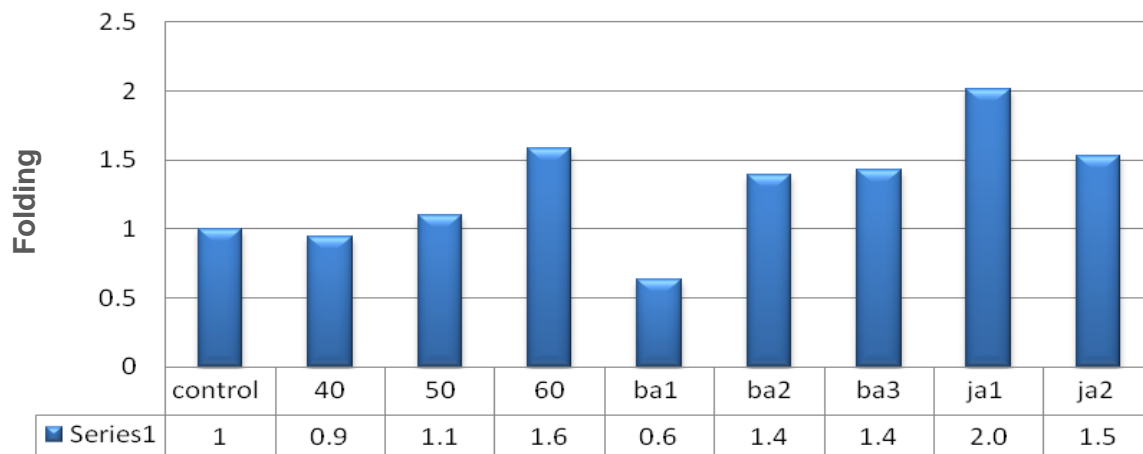


Figure 5. The folding levels of *tdc* gene expression between treated and untreated (control) *C. roseus*. 40, 50 and 60 = sucrose treatments (g/l); ba = benzyl adenine treatment; ja = jasmonic acid treatment.

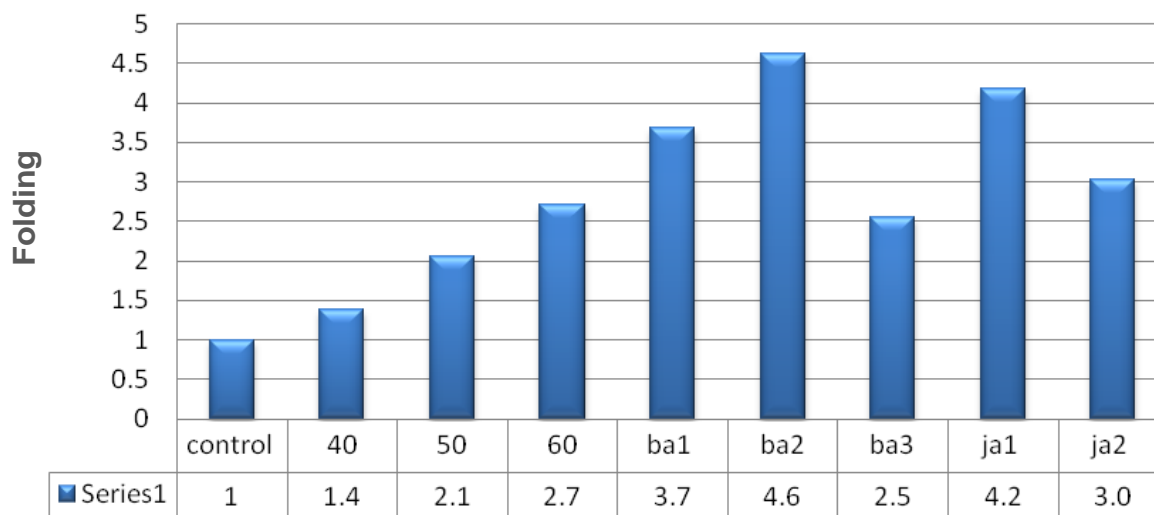


Figure 6. The folding levels of *cyp72A1* gene expression between treated and untreated (control) *C. roseus*. 40, 50 and 60 = sucrose treatments (g/l); ba = benzyl adenine treatment; ja = jasmonic acid treatment.

Transcriptional profiling of *tdc* gene under different treatments

Levels of *tdc* gene expression between treated and untreated (control) *C. roseus* determined by the comparative $\Delta\Delta C_T$ method are shown in Table 5 and Figure 5. Positive values of $\Delta\Delta C_T$ were detected in two cases only among the eight treatments: S1 (40 g/L sucrose) and ba1 (0.2 mg/L benzyl adenine) indicated down-regulation of this gene under these two treatments. Two-fold increase in gene expression of *tdc* was obtained when *C. roseus* callus was treated with 10 μ M jasmonic acid (ja1) and just only 1.5x value was obtained when callus was treated with 100 μ M (Ja2).

Transcriptional profiling of *cyp72A1* gene under different treatments

Table 6 and Figure 6 represent the folding levels of *cyp72A1* mRNA between treated and untreated (control) *C. roseus* calli. Negative $\Delta\Delta C_T$ values were obtained in all treatments indicating an up-regulation of *cyp72A1* gene transcription. The three sucrose treatments S1, S2 and S3 (40, 50 and 60 g/l, respectively) resulted in the increase in folding levels (1.4, 2.0 and 2.7, respectively). The *cyp72A1* gene seems to respond to sucrose treatments in a dose response manner. The maximum expression of *cyp72A1* gene (4.6x) was observed in ba2, when the callus was treated with 0.2 mg/l benzyl adenine.

jasmonic acid resulted in up-regulation of *cyp72A1* gene in its two concentrations: ja1 and ja2 (10 and 100 μ M, respectively). The folding levels were 4.1x and 3.2x, respectively.

Results of this study showed that the three genes respond differentially to the eight treatments. *cyp72A1* showed maximum folding of expression (4.2) between treated and untreated callus under ba2 treatment. This showed the influence of benzyl adenine in up-regulating this gene. The remaining genes represented comparable expression in all treatments. *str1* gene was up-regulated in all treatments except 4% sucrose treatment (0.7) and ja2 treatment (0.3), and with *tdc* gene, it was up-regulated in all treatments except 4% sucrose (0.9), while *cyp72A1* gene was up-regulated in all treatments.

Knowledge of the regulation of these biosynthetic genes will be helpful for metabolic engineering of terpenoid indole alkaloid productivity. Further studies would focus on investigating the correlation between the over expression (up-regulation) of the studied genes and the metabolic flow during TIAs biosynthesis in *C. roseus*.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Seasonal response of okra (*Abelmoschus esculentus* L. Moench) genotypes for okra yellow vein mosaic virus incidence

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One of the major limiting factors of okra is the incidence of okra yellow vein mosaic virus (OYVMV), its vector being whitefly. Infection of 100% plants in a field is very common and yield losses range from 50 to 94% depending on the stage of crop growth at which infection occurs. A total of 91 okra genotypes were evaluated in summer and rainy season of 2012 to 2013, out of which only 6 genotypes (IIHR123, IC90381, CI140982, IC141065, IIHR1, and Kavya) were found to have highly resistant disease reaction during both the season. The highest fruit yield in rainy and summer season was observed in the genotypes IC32855 (308 g/ plant) and IC99646 (212.3 g/plant), respectively. These genotypes could be used for further hybrid breeding programme due to their high yield and lower disease incidence for OYVMV.

Key words: Disease incidence, genotypes, okra, OYVMV, seasonal variation.

INTRODUCTION

Okra is one of the most popular vegetable crops cultivated throughout India for its tender green fruits. Okra requires a long and warm growing season for optimum growth and development. India is the largest producer of okra in the world with total area of 0.52 million hectares and production 6.26 million tones green pods, whereas productivity of the crop is 12.1 MT/ ha (Anonymous, 2012). Okra can be grown twice a year in the Indian plains (rainy season and summer season). The major limiting factor for its cultivation is the incidence of okra yellow vein mosaic virus (OYVMV) which is transmitted by whitefly (*Bemisia tabaci* Gen.) (Rana et al., 2006). This disease is caused by a complex, consisting of the monopartite begomovirus, okra yellow vein mosaic virus

(family: Geminiviridae) and a small satellite DNA β component (Jose and Usha, 2003). This disease and its insect vector cause heavy losses to okra by affecting the quality and yield of the fruits. Infection of 100% plants in a field is quite common and yield losses range from 50 to 94% depending on the stage of crop growth at which infection occurs (Sastry and Singh, 1974). The initial symptom on young leaves is a diffuse, mottled appearance. Older leaves have irregular yellow interveinal areas. Clearing of the small veins starts near the leaf margins, at various points, about 15 to 20 days after infection. Thereafter, the vein clearing develops into a vein chlorosis. The newly developed leaves exhibit an interwoven network of yellow vein, which enclose the green patches of the leaf.

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Table 1. Scale for classifying disease reaction in okra to OYVMV disease.

Symptom	Severity grade	Response value	Coefficient of infection	Reaction
Symptoms absent	0	0	0 – 4	HR
Very mild symptoms up to 25% leaves	1	0.25	4.1 – 9	R
Appearance of disease between 26 to 50% leaves	2	0.50	9.1 – 19	MR
Symptom between 51 to 75% leaves	3	0.75	19.1 – 39	MS
Severe disease infection at 75% leaves	4	1.00	39.1 – 69	S
Above 75% leaves	>4	>1.00	69.1 – 100	HS

Note: HR = Highly resistant, R = Resistant, S = Susceptible, H S = Highly susceptible, MR = Moderately resistant, MS = Moderately susceptible. Correlations between variables were tested for significance (Gomez and Gomez, 1984).

Fruits developing on infected plants have irregular yellow areas which follow a longitudinal alignment. Due to heavy infestation the fruits become malformed and reduced in size. The fruits are mostly yellow, small, tough and fibrous (Brunt et al., 1996). If plants are infected within 20 days after germination, their growth is retarded; few leaves and fruits are formed (Sastry and Singh, 1974). The extent of damage declines with delay in infection of the pathogens. Plants infected 50 and 65 days after germination suffer a loss of 84 and 49%, respectively (Sastry and Singh, 1974; Khan et al., 2005). With this severe production constraint in purview, the objectives of this study were to identify okra genotypes for resistance/ tolerance to OYVMV based on a percent disease incidence and coefficient of infection in okra under field conditions. The outcome of the study could open avenues for utilization of these genotypes in further hybrid breeding programme for OYVMV tolerance/resistance in okra.

MATERIALS AND METHODS

The present investigation was carried out using 91 diverse okra genotypes including 4 checks (Arka Anamika, Arka Abhay, VRO-6 and Pusa Sawani) collected from NBPGR, New Delhi, India and IIHR, Bengaluru, India, screened and evaluated at the research farm of the Department of Horticulture (Vegetable and Floriculture), Bihar Agricultural College, Bihar Agricultural University, Sabour, Bhagalpur (Bihar) in two different seasons, Summer and Rainy, 2012 to 2013. The recommended agronomic practices for raising a good okra crop were adopted. Observations were recorded on 12 economically important traits viz., days to first flowering, days to 50% flowering, first fruit picking, first flowering node, fruit length (cm), number of branch/ plant, plant height (cm), plant canopy width (cm), number of fruits/ plant, average fruit weight (g), fruit yield/ plant (g) and percent infestation of OYVMV. The soil of the plot was sandy loam in texture having good fertility, properly leveled and well drained. Scoring of OYVMV disease incidence was done on a scale of 0 to 4 (Table 1) at 15 days intervals (30, 45, 60 and 75 days) after sowing and PDI and CI value were calculated by the procedure coined by Banerjee and Kalloo (1987).

PDI (%) = (Number of infected plants / Total number of plants observed) × 100

CI (%) = PDI × RV

Where PDI = percent disease infection, RV= response value.

RESULTS AND DISCUSSION

Effect of season on growth and yield attributing traits

The effect of growing seasons (summer and rainy) was markedly observed through the mean performance of the crop itself. The summer crop was earlier than the rainy crop (the respective mean days to first flowering being 39.71 and 46.14 days after sowing, respective mean days to 50% flowering being 46.43 and 53.21 days after sowing, respective node number to first flower being 3.93 and 7.79 and respective days to first fruit picking being 49.21 and 53.36 days after sowing) (Table 2). IC33332 was the earliest genotype, whereas among checks Arka Anamika was the earliest. IC18536 was the latest genotype whereas Pusa Sawani among the checks was the most late. The effect of season was evident in yield and yield attributing characters also. In summer (Table 2) the mean yield per plant was recorded to be 154.57 g whereas in the rainy season it was massively increased to 247.85 g. Similar trends were observed in the various yield attributing traits, viz., number of fruits per plant, plant height, plant canopy and number of branches per plant which were lesser in summer and magnified in rainy season. However, the average fruit weight did not differ much over the seasons (12.20 g in summer and 12.21 g in rainy). During rainy season (Table 3) highest fruit yield was observed in the genotype IC32855 (308 g/ plant), whereas during summer season the same was recorded in the genotype IC99646 (212.3 g/plant). Among checks the maximum yield per plant was observed in the genotype Arka Anamika (rainy season: 253.6 g and summer season: 139.1 g) during both seasons. In summer season the highest average fruit weight was recorded in the genotype IIHR 113 (18.0 g), whereas in rainy season the same was observed in case of IC13995 (17.7 g). The number of fruits per plant was highest in IC128035 and IC31398 (19.6) in summer crop, whereas in rainy crop the highest was observed in IC18553 and IC18537A (25.4). Plant height was maximum in IC99646 in summer (110.0 cm) whereas in rainy season in the check VRO-6 (145.0 cm) closely followed by IC13995 (135.5 cm). Plant canopy in summer season was highest in IC99646 (90.0

Table 2. Mean performance of promising okra genotypes during summer season.

Genotype	Days to first flowering	Days to 50% flowering	First fruit picking	First flowering node	Fruit length (cm)	Number of branch/plant	Plant height (cm)	Plant canopy width (cm)	Number of fruits/plant	Average Fruit weight (g)	Fruit yield/plant (g)	OYVMV coefficient of infection (%)
IC99646	46	52	57	6	14.0	3.0	110.0	90.0	12.9	16.5	212.3	75.0
IC90219	38	43	47	2	15.0	3.0	50.0	60.0	18.8	10.8	204.1	75.0
IC33206A	36	42	46	2	15.0	2.0	50.0	55.0	15.1	13.4	202.5	5.0
IC111515	34	40	43	2	18.0	4.0	45.0	60.0	12.7	15.4	196.3	5.0
IC128035	39	46	52	4	15.0	2.0	40.0	50.0	19.6	9.6	188.4	16.7
IC31398	40	48	51	2	12.0	1.0	50.0	45.0	19.6	9.6	188.4	16.7
IC43741	32	38	44	3	11.0	3.0	60.0	68.0	12.9	12.8	165.7	16.7
IC43750	34	38	42	2	17.0	5.0	60.0	70.0	15.5	10.1	155.9	21.4
IIHR113	58	63	64	9	10.0	3.0	60.0	52.0	8.6	18.0	155.0	0.0
IC111321	37	41	46	3	14.0	1.0	45.0	55.0	17.5	8.7	151.8	0.0
Arka Anamika (c)	41	53	49	6	12.0	2.0	40.0	41.0	7.2	9.6	139.1	0.0
VRO-6 (c)	39	45	49	4	13.0	1.0	35.0	40.0	2.5	15.0	137.7	0.0
Arka Abhay (c)	38	48	44	4	10.0	3.0	60.0	90.0	10.3	11.1	118.4	5.0
Pusa Sawani (c)	44	53	55	6	9.9	4.0	78.0	67.0	12.0	10.2	115.4	75.0
General Mean	39.71	46.43	49.21	3.93	13.28	2.64	55.93	60.21	13.24	12.20	154.57	22.25
C.D. at 5%	13.92	15.10	13.23	4.57	5.58	2.62	41.08	33.72	10.68	6.41	112.68	63.28

Table 3. Mean performance of promising okra genotypes during rainy season.

Genotype	Days to first flowering	Days to 50% flowering	First fruit picking	First flowering node	Fruit length (cm)	Number of branch/plant	Plant height (cm)	Plant canopy width (cm)	Number of fruits/plant	Average Fruit weight (g)	Fruit yield/plant (g)	OYVMV coefficient of infection (%)
IC32855	48	54	55	10	11.3	4.0	95.0	87.0	24.5	12.6	308.0	45.0
IC18530	47	52	55	9	9.7	4.0	105.0	100.0	21.8	13.0	283.0	45.0
IC33332	40	44	49	7	10.7	4.0	125.3	99.0	23.8	10.9	259.0	80.0
IC13995	46	57	55	8	8.7	4.0	135.5	105.0	14.6	17.7	259.0	52.5
IC18536	52	58	56	8	7.4	4.0	119.0	90.0	24.3	10.6	256.3	25.0
IC18553	48	55	53	7	10.6	2.0	128.9	105.0	25.4	10.0	254.0	80.0
IC18542	46	52	55	9	9.9	3.0	132.0	80.0	18.5	13.6	251.8	25.0
IC15036	46	53	52	7	10.4	4.0	126.0	106.0	22.5	11.1	250.0	80.0
IC14845B	48	55	55	9	8.5	3.0	75.0	78.0	17.5	14.3	249.5	25.0
IC18537A	44	52	52	8	9.4	4.0	117.5	99.0	25.4	9.7	247.6	80.0
Arka Anamika (c)	40	52	46	6	8.6	5.0	100.6	80.0	18.0	14.1	253.6	20.0

Table 3. Contd.

VRO-6 (c)	43	46	52	8	8.0	3.0	145.0	105.0	20.8	11.7	243.0	45.0
Arka Abhay (c)	46	58	57	6	11.0	4.0	113.0	95.0	21.8	10.8	235.3	25.0
Pusa Sawani (c)	52	57	55	7	10.2	4.0	110.0	75.0	11.0	10.9	120.0	100.0
General Mean	46.14	53.21	53.36	7.79	9.59	3.71	116.27	93.14	20.70	12.21	247.85	51.96
C.D. at 5%	7.74	8.90	6.39	2.55	2.57	1.57	39.32	24.22	9.22	4.68	87.82	58.07

Table 4. Genotypic character association in okra during rainy and summer season.

Characters	Days to first flowering	Days to 50% flowering	First fruit picking	First flowering node	Fruit length (cm)	Number of branch/ plant	Plant height (cm)	Plant canopy width (cm)	Number of fruits/ plant	Average Fruit weight (g)	Fruit yield/ plant (g)
Days to first flowering	Rainy	0.746*	0.789*	0.312	-0.030	-0.247	-0.210	-0.270	-0.185	-0.130	-0.329
	Summer	0.945*	0.948*	0.884*	-0.500	-0.033	0.357	-0.055	-0.297	0.465	-0.074
Days to 50% flowering	Rainy		0.640*	-0.037	-0.032	0.047	-0.305	-0.273	-0.271	0.135	-0.237
	Summer		0.891*	0.906*	-0.625*	-0.071	0.314	-0.063	-0.376	0.330	-0.217
First fruit picking	Rainy			0.436	0.086	-0.305	-0.086	-0.073	-0.107	0.040	-0.088
	Summer			0.839*	-0.474	-0.112	0.407	-0.102	-0.184	0.430	0.023
First flowering node	Rainy				-0.085	-0.255	-0.235	-0.135	0.101	0.295	0.448
	Summer				-0.612*	0.049	0.372	0.030	-0.532	0.414	-0.326
Fruit length (cm)	Rainy					-0.060	-0.068	0.062	0.198	-0.308	-0.006
	Summer					0.201	-0.202	-0.067	0.384	-0.008	0.440
Number of branch/ plant	Rainy						-0.255	-0.192	-0.168	0.165	-0.019
	Summer						0.438	0.572*	0.008	0.161	0.226
Plant height (cm)	Rainy							0.625*	0.107	-0.121	-0.065
	Summer							0.765*	0.020	0.330	0.334
Plant canopy width (cm)	Rainy								0.503	-0.150	0.371
	Summer								0.086	0.148	0.307
Number of fruits/ plant	Rainy									-0.568*	0.652*
	Summer									-0.500	0.783*
Average Fruit weight (g)	Rainy										0.235
	Summer										0.109

* Significant at 5% probability levels.

cm), whereas in rainy season IC15036 had the greatest canopy width (106.0 cm). The number of branches per plant was highest in IC43750 (5.0) in summer, whereas in rainy season the highest

number of branches per plant was recorded in the check Arka Anamika (5.0). On the basis of correlation analysis during both the seasons (Table 4), days to first flowering, days to 50% flowering,

plant height and number of fruits per plant are significantly correlated. However, number of fruits per plant has significant negative correlation during rainy season. It was found that days to first

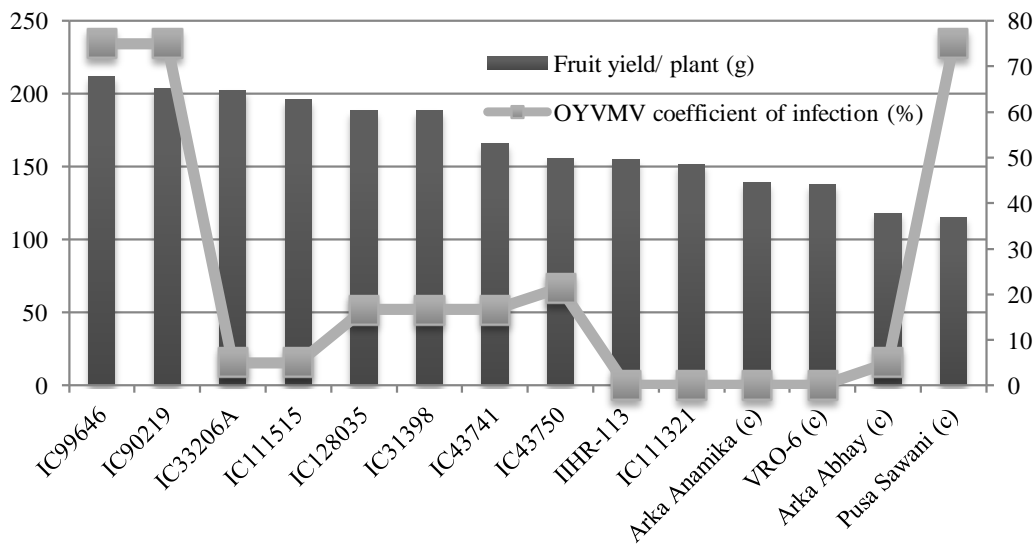


Figure 1. Performance of promising okra genotypes for yield and OYVMV infection during summer season.

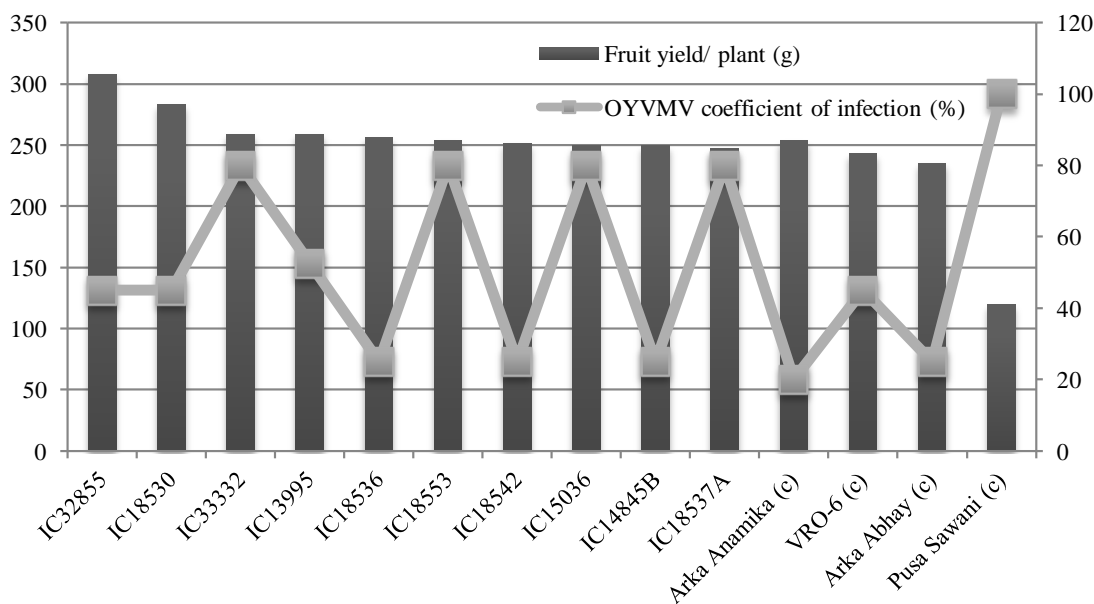


Figure 2. Performance of promising okra genotypes for yield and OYVMV infection during rainy season.

flowering, days to 50% flowering, days to first fruit picking via first flowering node; number of branches per plant via plant canopy width have positive significant correlation during summer season. Similar pattern of correlation was also accounted by (Dhankar and Dhankar, 2002; Das et al., 2013; Solankey et al., 2013).

Effect of season on OYVMV attributing traits

In the rainy season crop the intensity of OYVMV was very high due to the favourable environmental condition for the

vector, that is, whitefly and the virus (Das et al., 2013). The mean incidence of OYVMV in summer season (Figures 1 and 2) was low (22.25%) compared to the high incidence in rainy season (51.96%). Out of 91 okra genotypes, 45 genotypes were found to be highly resistant, 17 resistant, 10 moderately resistant, 08 moderately susceptible, 06 susceptible and rest 05 showed highly susceptible reactions during summer season (Table 5). However, only 6 genotypes were found to be highly resistant (IIHR123, IC90381, C1140982, IC141065, IIHR1, Kavya), 6 resistant, 5 moderately resistant, 14 moderately sus-

Table 5. Reaction of okra genotypes against OYVMV during both seasons (summer and rainy).

Disease Reaction	Summer Season, 2013		Rainy Season, 2013	
	Number of Genotypes	Name of Genotypes	Number of Genotypes	Name of Genotypes
Highly resistant	45	IC90381 (<i>A. tuberculatus</i>), IC140982 (<i>A. tetraphyllus</i>), IC141065 (<i>A. moschatus</i>), IIHR1 (<i>A. tetraphyllus</i>), IC15027, IC18537, IC13999A, IC14018, IC111321, IC90210, IC23628, IC18073A, IC18540, IC18530, IC90212, IIHR123, IIHR110, IIHR113, IC43742, IC23594, IC32298, IC117116, IC32855, IC18541A, IC27875, IC32850, IC33323A, Arka Anamika, IIHR112, IIHR129, IIHR53, IIHR115, IC14909, IC16566, IC45727, IC26375, IC18532, IC18975, IC18537A, IC15036, VRO-6, Kavya, RBH-18, IC15435, IIHR120.	06	IIHR123, IC90381 (<i>A. tuberculatus</i>), IC140982 (<i>A. tetraphyllus</i>), IC141065 (<i>A. moschatus</i>), IIHR1 (<i>A. tetraphyllus</i>), Kavya
Resistant	17	IC18960, IC13995, IC22237, IC18542, IC128065, IC111515, IC33206A, Arka Abhay, IC18536, IC43745, IC18960B, IC18553, IC90170, IC99709, IIHR43, IC111520, IC16262A	06	IC15435, IIHR113, IIHR120, IIHR53, IIHR129, RBH-18
Moderately resistant	10	IC45723, IC14845B, IC90298, IC31398, IC43743, IC14600, IC43741, IC31037A, IC22283, IC43149	05	IC111520, IC16566, IC33206B, IIHR128, IC99646
Moderately susceptible	08	IC43751, IC43750, IC128037, IC43746D, IC90219, IC128035, IC11537, IC128071	14	Arka Abhay, Arka Anamika, IC128049, IC14845B, IC16262A, IC18532, IC18536, IC18541A, IC18542, IC22283, IC32850, IC43149, IIHR112, VRO-6
Susceptible	06	IC33332, IC22282, IC33332, IC43741, IC44526, IIHR116	29	IC11537, IC111515, IC128035, IC13999A, IC18073A, IC18530, IC31037A, IC32855, IC33206A, IC43741, IC90219, IIHR 43, IIHR110, IC 43750, IC 90212, IC128071, IC13995, IC14018, IC14026, IC22282, IC26375, IC43742, IC43743, IC43745, IC45723, IC90170, IC90210, IC99709, IC18960B
Highly susceptible	05	IC14026, IC128049, IC99646, IC33206B, Pusa Sawani	31	IC128037, IC117116, IC14600, IC15036, IC18537A, IC18540, IC18553, IC18975, IC22237, IC23594, IC27875, IC32298, IC33323A, IC33332, IC43741, IC44526, IC45727, IC128065, IC15027, IC18960, IC43746D, IC43751, IC111321, IC14909, IC18537, IC23628, IC31398, IC90298, IIHR115, IIHR116, Pusa Sawani

ceptible, 29 susceptible and rest 31 showed highly susceptible reaction during rainy seasons. Only six genotypes were found to have common highly resistant response against OYVMV during both the season, that is, IIHR123, IC90381, IC140982, IC141065, IIHR1 and Kavya. However, the check variety Pusa Sawani was found to have 100% disease incidence during rainy season and 75%

incidence in summer season. Among the six highly resistant genotypes four (IC90381: *Amaranthus tuberculatus*; IC140982: *Amaranthus tetraphyllus*; IC141065: *Amaranthus moschatus*; IIHR1: *A. tetraphyllus*), are from wild back ground.

On the basis of two season research work, it was observed that the okra crop has more yield potential during rainy season but this season was

not only favours growth and development of crop but also OYVMV infestation. None of the genotypes showed higher yield potential coupled with high resistance to OYVMV during rainy season (Figures 1 and 2). The check varieties were also more prone to OYVMV during the rainy season than in summer. The incidence of the disease in a particular genotype also varied from season

to season, probably due to the influence of the environmental conditions. It is worth mentioning that Singh (1990) reported that hot weather with little rainfall was favourable for development of OYVMV and also for multiplication of the vector *Bemisia tabaci*. Earlier, other reports indicated that the incidence of OYVMV disease was higher during the rainy season when relative humidity was very high which support our findings (Sangar, 1997; Bhagat et al., 2001; Chattopadhyay et al., 2011; Das et al., 2013).

Conclusion

It may be concluded that there was significant effect of season on yield and yield attributing traits as well as incidence of OYVMV in okra. While summer crop is early, higher values for yield attributing traits and yield were recorded in rainy crop. Rainy season favours incidence of OYVMV. However, this season also favours the morphological and reproductive phase of okra. Moreover, rainy season crop has more yield potential than summer season crop. From the large number of genotypes evaluated under this study, the genotypes IIHR123, IC90381, CI140982, IC141065, IIHR1, Kavya, IC32855 and IC99646 and check variety Arka Anamika had high potential and lower incidence for OYVMV. Hence, these genotypes could be utilized for further breeding programmes.

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Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Analyses of soil cadmium and copper contents on a *Domérien* soil series of Burgundy in France

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The aim of this study is to determine the availability of cadmium (Cd) in the soil of Yonne district, Burgundy, France. Soil samples were collected from surface-ploughed layer in fields across the southern part of the Yonne district, Burgundy, France. Some results analysing soil Cd and Cu contents on *Domérien* region of Burgundy has been presented. This is to know the relationship between extracting time and extracted Cd and Cu contents from the two sites' soils (Dubloc, Bierry) in the *Domérien* soil series. A total of 68-107% Cd and 20-28% Cu were extracted from the soil in 1440 min. The two soils were acidic, and around 70% of Cd contents were extracted in 20 min of extraction, while around 65% of Cu contents were extracted in 30 min of extraction. The Cd extraction was blocked at the mid-extraction time, while the Cu extraction kept on increasing up to the end of the extraction time. Cadmium extraction increased with increase in pH value in the present experiment. Copper content shows same tendency with Cd.

Key words: Atomic absorption spectrophotometer, cadmium availability, copper extraction, *Domérien* soil series, extracting time, soil analyses.

INTRODUCTION

Since cadmium (Cd) occurs in zinc (Zn), lead (Pb) and copper (Cu) ores, the mining and smelting of these metals, particularly Zn and Cu, are now major sources of local environmental pollution by Cd (Piotrowski and Coleman, 1980). Cadmium content of the surface soil can vary greatly (0.14-3.51 mg Cd/kg soil) depending on soil series and sampling site (Mench et al., 1997). On

forest ecosystems, cadmium chloride concentrations in Serbian spruce [*Picea omorika* (Panc.) Purkyne] of up to 0.1 mM did not inhibit germination, while 1 mM concentration inhibited germination and the activities of catalase, superoxide dismutase and peroxidase

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(Prodanovic et al., 2012). As a result, Cd lowered the leaf contents of the photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) in common bean (*Phaseolus vulgaris* L. cv. Nebraska) plants (Aldoobie and Beltagi, 2013), and Cd stress enhanced antioxidant enzymes, guaiacol pero-xidase, glutathione reductase, ascorbate peroxidase and polyphenol oxidase in concentration and time dependent manner in leaves of hyacinth bean (*Lablab purpureus*) (D'Souza and Devaraj, 2013). The Cd concentrations determined by EDXRF (high-sensitivity energy-dispersive X-ray fluorescence spectrometry) for ten samples of rice containing Cd at concentrations ranging from 0.05 to 2.42 mg/kg agreed with the values determined by atomic absorption spectrometry (Honma et al., 2013).

A field study was undertaken in five soil series (*Domérien*, *Carixien*, *Terres noires*, *Sols marron*, *Aubes*) from the southern part of the Yonne district, Burgundy, France (Mench et al., 1997). This area has various soil series with either low or high geochemical Cd content in the topsoil. The wheat Cd content on 5 soil series were reported as follows (Mench et al., 1997); (*Domérien* < *Carixien* < *Terres noires* < *Sols marron* < *Aubes*). After the work of Mench et al. (1997), we took the work of knowing the relations between extracting time and extracted Cd and Cu contents from the two soils in the *Domérien* soil series. The absorbance of the two mineral elements was determined by atomic absorption spectrophotometry (Arnaud, 1998; Park et al., 2008) and pH was determined. The aim of this study was to determine the availability of Cd (Etherington, 1982; Alloway, 1990) thus, some results analysing soil Cd and Cu contents on *Domérien* region of Burgundy (*Bourgogne*) in France are hereby presented.

MATERIALS AND METHODS

Soil samples were collected on 0.3 m² area with a spade from the 0 to 0.25 m surface-ploughed layer in fields at 2 sites on *Domérien* region from the Yonne district of Burgundy, France (Mench et al., 1997). Soil samples were air-dried, 2 mm sieved and re-homogenized. And the soil samples from the *Domérien* soil series were used for the analyses of Cd and Cu contents. The names of these soils are Dubloc and Bierry.

The method for extraction was giving by Lebourg (1996) and Ghestem (1997). The time for extraction was from 5, 10, 20, 30, 60, 300, up to 1 440 min (24 h) for the two soils. And the reagent for extraction was 0.05 M EDTA (ethylene diamine tetraacetic acid) on the form of Na₂H₂EDTA and the soil was extracted by an agitator into a polyethylene bottle (volume around 50 ml) and the extracted solution was filtrated with a Millipore system (radius of membrane, Ø = 0.45 µm). The weight of soil and the amount of EDTA were 10 g and 30 ml, respectively. The experiment was carried out on *Laboratoire de Chimie Analytique* of *Institut National Agronomique Paris-Grignon* (INA P-G) in France from February 10, 1998 to January 20, 1999. The Cd and Cu contents of the extracted soil solution were determined by air-acetylene flame atomic absorption spectrophotometer (AAS) (model: VARIAN SPECTRAA 250 PLUS), and their wave lengths were 228.8 µm and 324.8 µm, respectively. A background correction mode was used for Cd analysis (Park et

al., 2008), but was not used for Cu analysis (Pinta et al., 1979).

RESULTS AND DISCUSSION

Table 1 shows the absorbance, cadmium (Cd) content and pH values of extracted soil solution on natural logarithm value of the extracting time on Dubloc soil, and Table 2 shows these values on Bierry soil on the *Domérien* soil series on a background correction mode.

The two soils were acidic, and the data of absorbance value was different between Tables 1 and 2. It is natural because the condition of each analysis was different. The quantity of the extracted Cd increased with the advance of extracting time (5-1440 min), and around 70% of Cd contents were extracted on 20 min of extraction. There were several factors responsible for the decision of Cd content with an atomic absorption spectrometer, for example, pH, concentration of EDTA, duration of extraction or ratio of weight/volume of solution (Ghestem, 1997).

Table 3 shows the absorbance, Cu content and pH values of extracted soil solution on natural logarithm value of the extracting time on Dubloc soil, and Table 4 shows these values on Bierry soil on the *Domérien* soil series not on a background correction mode. Table 3 and Table 4 show the variance of the absorbance and the extractable Cu content on the 2 French soils with an atomic absorption spectrometer. The absorbance value was different from that of Kim et al. (2000), and the data between Table 3 and Table 4 was also different. Pinta et al. (1979) wrote that there are some factors which have effect on the changes of absorbance of Cu, for example, temperature, associated anion and interaction with other minerals. The quantity of the extracted Cu increased with the advance of extraction, and around 65% of Cu contents were extracted on 30 min of extraction.

Table 5 shows the comparison of soil Cd values on the *Domérien* soil series with the data of Mench et al. (1997). The total Cd, which has been obtained on humid digestion (mineralisation) with utilising HNO₃, HCl and HF (Mench et al., 1997), was 0.14 (mg Cd/ kg soil) on Dubloc soil and 0.22 (mg Cd/ kg soil) on Bierry soil, respectively. And on Table 5, there was a significant difference in Cd content between those on a background correction mode and those not on a background correction mode. As written previously it is natural, because the condition of each analysis differs. There was no significant difference between total Cd (Mench et al., 1997) and Cd extractable on our present results (68-107% of total Cd).

Table 6 shows the comparison of soil Cu values on the *Domérien* soil series with the data of Mench et al. (1997). The total Cu, which has been obtained on humid digestion (mineralisation) by utilising HNO₃, HCl and HF (Mench et al., 1997), was 10.4 mg Cu/ kg soil on Dubloc and 15.7 mg Cu/ kg soil on Bierry, respectively. There were big differences between total Cu (Mench et al,

Table 1. Absorbance, cadmium(Cd) content and pH values of extracted soil solution on natural logarithm value of the extracting time on Dubloc soil on the Domérien soil series.

Extracting time (min) *	1**	5	10	20	30	60	300	1440
Value of natural logarithm (log e)	0.000	1.609	2.302	2.995	3.401	4.094	5.703	7.272
Absorbance (x 0.001) ***		1	2	3	3	3	4	4
		1	2	3	3	4	5	4
		1	2	3	3	4	5	4
		1	2	3	3	3	4	4
		2	1	2	3	3	5	4
		1	2	3	3	4	4	4
		1	2	3	3	4	5	4
		2	2	3	3	4	4	4
Mean absorbance		1.2	1.8	2.8	3.0	3.6	4.5	4.0
± Standard deviation (x 0.001)		± 0.4	± 0.3	± 0.3	± 0.0	± 0.5	± 0.5	± 0.0
Absorbance per 1440 min (%)		30	45	70	75	90	112	100
Cd content (mg/kg soil)	0.00	0.03	0.06	0.09	0.09	0.12	0.15	0.15
pH (measured on 21 Dec 1998)	4.64 ****	4.07	4.07	4.09	4.11	4.16	4.34	4.54

*, 10 g soil + 30ml EDTA, and the samples were agitated and filtrated on 17–18 december 1998; **, the number, 1, was used instead of 0 in order to calculate the natural logarithm value of 0; ***, Analysed with an atomic absorption spectrophotometer on a background correction mode; ****, the value of pH by EDTA (0.05 M).

Table 2. Absorbance, cadmium (Cd) content and pH values of extracted soil solution on natural logarithm value of the extracting time on Bierry soil on the Domérien soil series.

Extracting time (minutes)*	1**	5	10	20	30	60	300	1440
Value of natural logarithm (log e)	0.000	1.609	2.302	2.995	3.401	4.094	5.703	7.272
Absorbance (x 0.001) ***		4	6	8	10	10	10	14
		5	8	9	9	10	11	14
		6	8	8	9	10	12	14
		5	7	10	7	8	10	14
		6	7	10	10	10	11	11
		5	8	10	9	9	9	13
		4	5	9	8	10	10	14
		4	6	8	8	8	11	11
Mean absorbance		4.8	6.8	9.0	8.7	9.3	10.5	13.1
± Standard deviation (x 0.001)		± 0.8	± 1.1	± 0.9	± 1.0	± 0.9	± 0.9	± 1.3
Absorbance per 1440 minutes (%)		36	51	68	66	70	80	100
Cd content (mg /kg soil)	0.00	0.06	0.06	0.09	0.09	0.09	0.12	0.15
pH (03 nov 1998)		3.86	3.80	3.84	3.84	3.87	3.98	4.21
pH (06 nov 1998)		4.17	4.17	4.14	4.19	4.19	4.28	4.50

*, 10 g soil + 30 ml EDTA, and the samples were agitated and filtrated on 2-3 november 1998 ; **, the number, 1, was used instead of 0 in order to calculate the natural logarithm value of 0; ***, Analysed with an atomic absorption spectrophotometer on a background correction mode.

Table 3. Absorbance, copper(Cu) content and pH values of extracted soil solution on natural logarithm value of the extracting time on Dubloc soil on the Domérien soil series.

Extracting time (min) *	1**	5	10	20	30	60	300	1440
value of natural logarithm (log e)	0.000	1.609	2.302	2.995	3.401	4.094	5.703	7.272
Absorbance (x 0.001) ***		12	12	18	20	21	26	30
		11	11	18	20	21	26	30
		12	11	17	20	21	26	31
		12	12	17	20	21	26	30
		11	12	18	20	21	26	30
		11	11	18	21	21	26	30
		11	12	17	20	20	26	30
		11	12	17	20	21	26	30
Mean absorbance		11.3	11.6	17.5	<u>20.1</u>	20.8	26.0	30.1
± Standard deviation (x 0.001)		± 0.5	± 0.5	± 0.5	<u>± 0.3</u>	± 0.3	± 0.0	± 0.3
Absorbance per 1440 minutes (%)		37	38	58	<u>66</u>	69	86	100
Cu content (mg/kg soil)	0.0	0.9	0.9	1.5	1.5	1.8	2.1	2.4
pH (measured on 21 dec 1998)	4.64 ****	4.07	4.07	4.09	4.11	4.16	4.34	4.54

*, 10 g soil+30ml EDTA, and the samples were agitated and filtrated on 17–18 December 1998; **, the number, 1, was used instead of 0 in order to calculate the natural logarithm value of 0; ***, Analysed with an atomic absorption spectrophotometer not on a background correction mode; ****, the value of pH by EDTA (0.05 M).

Table 4. Absorbance, copper(Cu) content and pH values of extracted soil solution on natural logarithm value of the extracting time on Bierry soil on the Domérien soil series.

Extracting time (min) *	1**	5	10	20	30	60	300	1440
Value of natural logarithm (log e)	0.000	1.609	2.302	2.995	3.401	4.094	5.703	7.272
Absorbance (x 0.001) ***		43	92	106	108	115	141	166
		42	92	106	110	116	143	163
		42	94	106	109	117	144	167
		42	89	104	108	114	137	160
		42	90	104	108	118	141	170
		41	91	106	108	115	144	174
		42	92	106	108	116	142	176
		42	92	107	108	117	141	173
Mean absorbance		42.0	91.5	105.6	108.3	116.0	141.6	168.6
± Standard deviation (x 0.001)		± 0.5	± 1.5	± 1.0	± 0.7	± 1.3	± 2.2	± 5.6
Absorbance per 1440 minutes (%)		24	54	62	64	68	83	100
Cu content (mg /kg soil)	0.0	1.2	2.4	3.0	3.0	3.3	3.9	4.5
pH (03 nov 1998)		3.86	3.80	3.84	3.84	3.87	3.98	4.21
pH (06 nov 1998)		4.17	4.17	4.14	4.19	4.19	4.28	4.50

*, 10 g soil +30 ml EDTA, and the samples were agitated and filtrated on 2–3 November 1998; **, the number, 1, was used instead of 0 in order to calculate the natural logarithm value of 0; ***, Analysed with an atomic absorption spectrophotometer not on a background correction mode.

1997) and those of our present results (20-28% of total Cu). But the range of this decrease was not as large as

that of Cd contents as shown in Table 5. Ghestem (1997) utilized an acidic pH and a neutral pH conditions, and the

Table 5. Comparison of soil cadmium(Cd) values on the Domérien soil series with the data of Mench et al. (1997) (mg Cd/ kg soil).

Soil series	Place	Cd extractable 2)*	Cd extractable 3)*	Cd extractable 4)**	Cd 3)/total Cd (%)	Cd 4)/ total Cd (%)
Domérien	Dubloc	0.21	0.12	0.15	85	107
"	Bierry	0.24	0.15	0.15	68	68

1) The total Cd, which has been obtained on humid digestion (mineralisation) with utilising HNO₃, HCl and HF (Mench et al, 1997), was 0.14 (mg Cd/ kg soil) on Dubloc soil and 0.22 (mg Cd/ kg soil) on Bierry soil, respectively.

2) 10 g soil and 30 ml EDTA of 0.05 mol, it was extracted and filtrated on 19-20 october 1998. The value was obtained with an atomic absorption spectrophotometer not on a background correction mode, and the duration of extraction, it took 14 minutes and 34 minutes for Dubloc and Bierry soil, respectively;

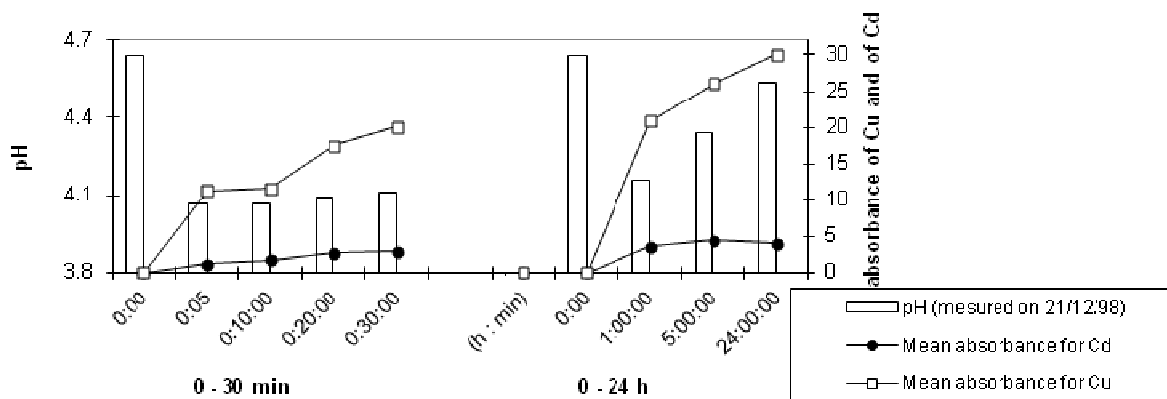
3) The same method with that of 2), but the sampels were measured with an atomic absorption spectrophotometer on a background correction mode;

4) Extraction of Dubloc soil shown on Table 1 (17–18 december 1998) and of Bierry soil on Table 2 (2-3 november 1998) during 1440 min, and it was analysed on a background correction mode; *, One sample ; **, One sample with 8 repeats.

Table 6. Comparison of soil copper(Cu) values on the Domérien soil series with the data of Mench et al.(1997) (mg Cu/ kg soil).

Soil series	Place	Cu extractable 2) *	Cu extractable 3) **	Cu 2)/total Cu(%)	Cu 3)/total Cu(%)
Domérien	Dubloc	2.1	2.4	20	23
"	Bierry	4.2	4.5	26	28

1) The total Cu, which has been obtained on humid digestion (mineralisation) with utilising HNO₃, HCl and HF (Mench et al, 1997), was 10.4 (mg Cu/ kg soil) on Dubloc and 15.7 (mg Cu/ kg soil) on Bierry, respectively. 2) 10 g soil and 30 ml EDTA of 0.05 mol, it was extracted and filtrated on the period of 19-20 october 1998. The value was obtained with an atomic absorption spectrophotometer without background correction, and the duration of extraction, it took 14 and 34 min for Dubloc and Bierry soil, respectively, 3) Extraction of Dubloc soil shown on Table 3 (17–18 december 1998) and of Bierry soil on Table 4 (2-3 november 1998) during 1440 min, and it was analysed on not background correction mode; *, One sample; **, One sample with 8 repeats.

**Figure 1.** Absorbances of Cd and of Cu on Dubloc (Domerien) soil with an atomic absorption spectrophotometer.

Cd content in the edible parts of vegetable species decreased with the increase of soil pH (Togami et al., 2011). In our work, Figure 3 shows the Cd content and pH value of extracted soil solution on natural logarithm value of the extracting time on Dubloc soil on the Domérien soil series. These data were taken from Table 1. The Cd contents on 20 min (4th) and 30 min extraction (5th) were stable. And Figure 4 shows the Cu content and pH value of extracted soil solution on natural logarithm value of the extracting time on Dubloc soil on the Domérien soil series.

These data were taken from Table 3. Here, the Cu con-

tents on 5 min (2nd) and 10 min (3rd), 20 min (4th) and 30 min extraction (5th) were stable. Figure 1 shows the absorbances of Cd and of Cu on Dubloc (Domerien) soil with an atomic absorption spectrophotometer, and Figure 2 shows the absorbances of Cd and of Cu on Bierry (Domerien) soil with an atomic absorption spectrophotometer.

Conclusion

The Cd content increased on higher pH range when

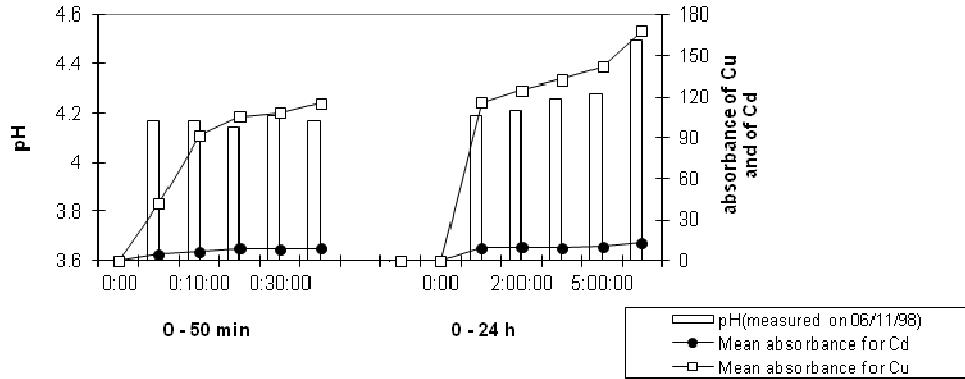


Figure 2. Absorbances of Cd and of Cu on Bierry (Domerien) soil with an atomic absorption spectrophotometer.

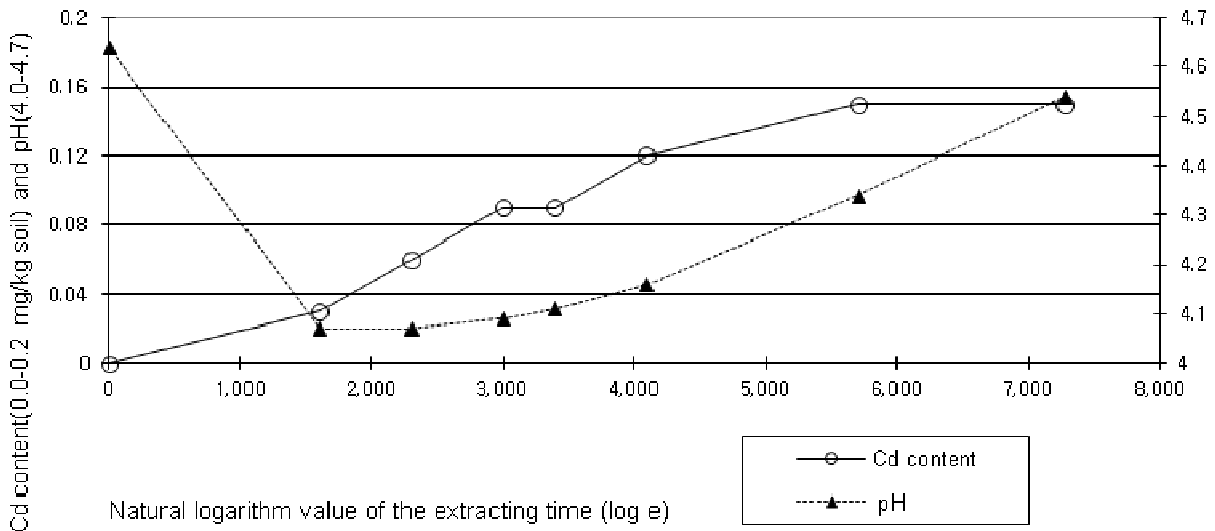


Figure 3. Cadmium(Cd) content and pH value of extracted soil solution on natural logarithm value of the extracting time on Dubloc soil on the Domérien soil series (These data were taken from Table 1).

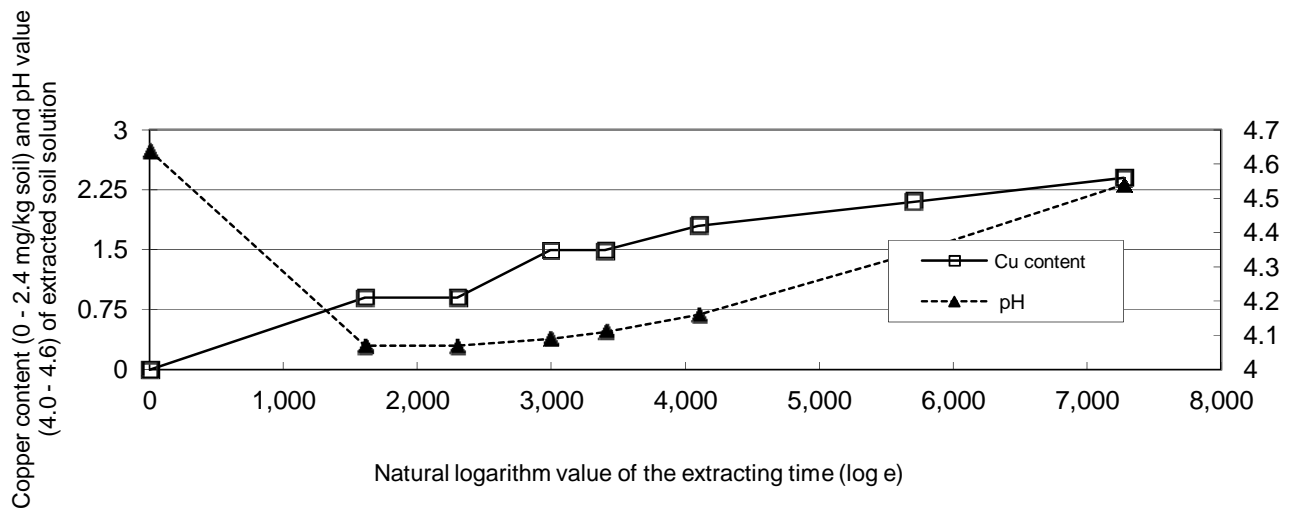


Figure 4. Copper(Cu) content and pH value of extracted soil solution on natural logarithm value of the extracting time on Dubloc soil on the Domérien soil series (These data were taken from Table 3)

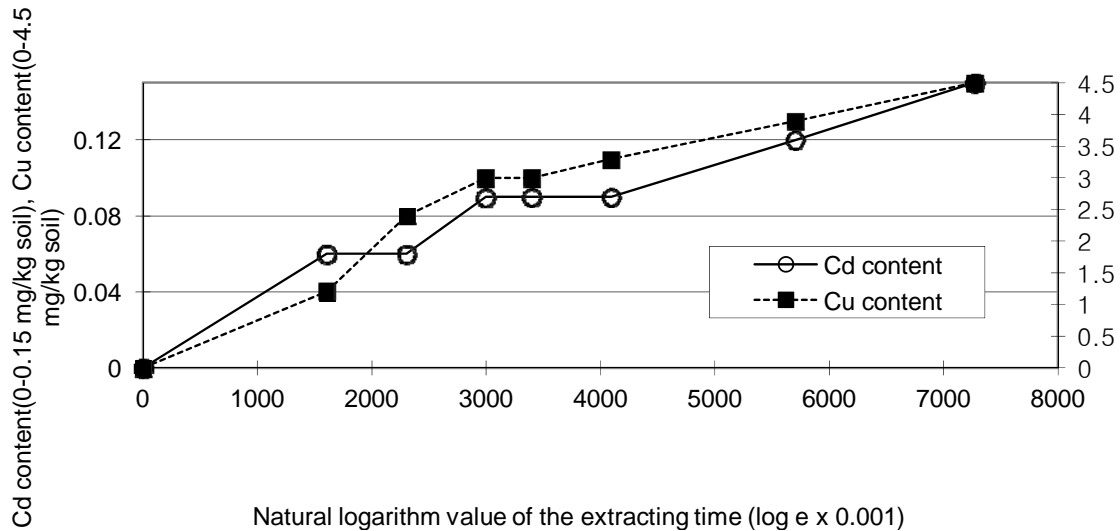


Figure 5. Cadmium(Cd) and copper(Cu) contents of extracted soil solution on natural logarithm value of the extracting time on Bierry soil on the Domerien soil series (These data were taken from Table 2 and Table 4).

varied in the present experiment. The Cu content showed same tendency with Cd, while the (Cu extractable/Cu total) ratio was significantly lower value of 20-28% than that value of the Cd ratio (Cd extractable/Cd total) of 68-107%. The Cd content was blocked in the middle of extracting time, while the Cu content kept continuing increasing upto the end of the extracting time. Therefore, the soil Cd could have a stronger effect on grain Cd content than those of Cu content.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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continuous reading of the manuscript.

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Full Length Research Paper

Identification of virus isolates inducing mosaic of sugarcane in Makarfi Local Government Area of Kaduna State, Nigeria

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Sugarcane mosaic disease caused by sugarcane mosaic virus (SCMV), Johnsongrass mosaic virus (JGMV), maize dwarf mosaic virus (MDMV) and sorghum mosaic Virus (SrMV) is an economically important viral disease of sugarcane worldwide. Field survey was conducted to assess the presence of the viruses involve in mosaic disease of sugarcane in Makarfi Local Government Areas of Kaduna State (Northern Guinea Savannah), Nigeria. A range of symptoms were observed on the infected land races from the pale green stripes to yellow chlorotic stripes on a dark green background. The purple land race ("Bakarkwama") was highly susceptible followed by green land race ("Bahausa") and the least infected was the white land race ("fararkwama"). 63 symptomatic and asymptomatic sugarcane leaves and stem juice extract from 14 villages of Makarfi L.G.A. were screened for the four viruses using DAS and TAS ELISA methods. SCMV, MDMV and SrMV were detected from both symptomatic and asymptomatic sugarcane leaf samples whereas JGMV was not detected in the locations sampled. SCMV isolate has the highest incidence (83%) from all the locations followed by SrMV (10%) and MDMV (5%) isolates. Mixed infections of the three viruses were also detected in some samples. This is the first report of identification of virus isolates inducing sugarcane disease in one of the major sugarcane producing areas of the Northern Guinea Savannah of Nigeria.

Key words: Sugarcane, SCMV, MDMV, SrMV, TAS-ELISA, DAS-ELISA.

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.), belongs to the family Poaceae, it is a high value cash crop in many parts of the world. It is an old energy source for humans, produced on commercial basis and is either chewed or use in the production of brown and refined sugar as well as ethanol. Virus is one of the major limiting factors in many sugarcane growing areas of the world (Grisham et

al., 2013). Mosaic in sugarcane is a member of potyvirus genus of the family potyviridae consisting of four distinct viruses based on serological properties; coat protein and genome sequence (Shukla et al., 1992) and is one of the most common and economically important viruses in sugarcane cultivars causing severe effect on sugarcane production worldwide. Forty (40) percent yield loss due to

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mosaic has been reported in Australia (Croft et al., 2000).

In South Africa, SCMD brought sugar industry to its knees (Anon, 1980). Under conditions of severe SCMV infection, reduction in sucrose yield up to 42% has been reported in susceptible varieties in South Africa (Bailey and Fox, 1987). Makarfi lies at 11° 22' N, 7° 52' E, it has 2, 520 sq miles and has a long history of sugarcane cultivation in northern Nigeria. Makarfi is blessed with abundant farmland and the major cash crop is sugarcane which is exported outside the state and country. Sugarcane is cultivated in 80% of the villages and three land races of chewing cane are grown. As common to areas where sugarcane is cultivated, sugarcane mosaic disease has caused great economic losses to farmers in all the areas *vis* reduced palatability, marketability and yield. Yang and Mirkov (1997) used genome-based technique to differentiate strains of SCMV and SrMV in Texas, USA. Balamuralikrishnam et al. (2004) used both genome-based and antibody-based techniques to detect SCMV in India. Mohammad and Behzad (2009) used antibody based technique and detected SCMV in Iran. However, in Nigeria, little work has been done on identification of sugarcane mosaic disease. Wada et al. (1999), reported SCMV incidence of 6% based on sap inoculations on susceptible maize varieties from the Southern Guinea Savannah Zone of Nigeria.

As these viruses are transmissible through infected seed canes, they pose the risk of accidental introduction into previous disease-free regions. The four distinct viruses induce similar pale green and yellow chlorotic stripes symptoms on leaf blade and white stripe on stem in infected sugarcane and are indistinguishable based on the visible symptoms. Furthermore, symptom expression may also be confused for environment disorders, as they both cause disruption in plant metabolism. These necessitate the use of antibody-based technique to diagnose the disease. The present paper reports for the first time that, the identification of the virus isolates causing mosaic disease of sugarcane in Makarfi, which is the major sugarcane producing areas of Kaduna State, (Northern Guinea Savannah) Nigeria.

MATERIALS AND METHODS

Survey for sugarcane viruses in sugarcane growing areas of Makarfi Local Government Area (L.G.A)

Survey for sugarcane viruses was conducted in 14 major sugarcane growing villages in Makarfi Local Government (Figure 1). The range of symptoms observed on both leaves and stem were recorded on the three main land races of chewing type sugarcane. A total of 63 samples comprising of both symptomatic and asymptomatic leaves were collected from ratoon and seedcane fields employing systematic sampling methods from May to October 2012. In each case, both symptomatic and asymptomatic leaves were collected from the youngest leaves and put in polythene bag, placed on icebox and stored at -20°C in freezer and some under calcium chloride. Coordinates were also recorded at each site using GPS. Each sample was later tested for presence of SCMV and

JGMV by DAS-ELISA and for MDMV and SrMV by TAS-ELISA.

DAS ELISA for the detection OF SCMV and JGMV

Antibodies to SCMV, JGMV and positive control were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Germany. 20 µl of purified IgG was diluted (1:1000) in 20 ml coating buffer (1.59 g/dm³ sodium carbonate + 2.93 g/dm³ sodium bicarbonate + 0.2 g/dm³ sodium azide). Wells of microtitre plates (Nunc) were coated with 200 µl of IgG and incubated at 37°C for 4 h. Plates were then washed thrice in PBST and tapped dry on tissue paper. Leaf tissues were homogenized separately using sterilized pestle and mortar at ratio of 1 g leaf tissue per 4 ml extraction buffer [PBST+2% PVP (Polyvinyl pyrrolidone)]. 200 µl aliquots of the test sample was added to duplicate wells and incubated at 4°C overnight. Plates were washed thrice as above with PBST and 200 µl anti-virus conjugate was added to each well, incubated at 37°C for 4 h and washed as above. Freshly prepared substrate [10 mg p-nitrophenyl phosphate in 10 ml substrate buffer (97 ml/dm³ diethanolamine + 600 ml/dm³ distilled water + 0.2 g/dm³ sodium azide)] was added (200 µl) to each well and incubated at room temperature. Colour change was recorded by visual observation. Absorbance (A_{405nm}) values were recorded in a microplate reader (optic ivymen system 2100 c) after 1 h at room temperature and overnight at 4°C. A_{405nm} values greater than two times that of the healthy control and were considered positive.

TAS ELISA for the detection of MDMV and SrMV

Plates were coated with antisera to MDMV and SrMV in coating buffer as recommended by the manufacturer (1:1000) and incubated at 37°C for 4 h. Plates were then washed thrice in PBST. Unbound spaces were blocked with 2% skimmed milk (sigma U.S.A.) in PBST and incubated at 37°C for 30 min. Test samples were prepared as described for DAS ELISA above and 200 µl were added to the wells and incubated at 4°C overnight. Monoclonal antibody at 1:1000 in conjugate buffer was added to each well after washing as above and incubated at 37°C for 4 h. Plates were washed and 200 µl anti-virus conjugate was added to each well and incubated at 37°C for 2 h. The plates were washed in PBST. Freshly prepared substrate [10 mg p-nitrophenyl phosphate in 10 ml substrate buffer (97 ml/dm³ diethanolamine + 600 ml/dm³ distilled water + 0.2 g/dm³ sodium azide)] was added (200 µl) to each well and incubated at room temperature. Colour change was recorded by visual observation. A_{405nm} values were recorded in a microplate reader (optic ivymen system 2100c). Absorbance values greater than two times that of the healthy control and were considered positive. Data recorded were analyzed using analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) was used to separate the mean.

RESULTS

Survey for sugarcane mosaic disease

SCMD symptoms was observed in all the villages visited exhibiting a range of symptoms especially on the leaves and stem. The symptoms range from pale green stripes to yellow chlorotic stripes on a dark green background on the leaves while white stripes were observed on the stem Figure 2. The farmers locally call it "Mamar" or "Maizabuwa" based on the symptoms. The Bakarkwama was found to be the most susceptible (76% disease incidence) and the

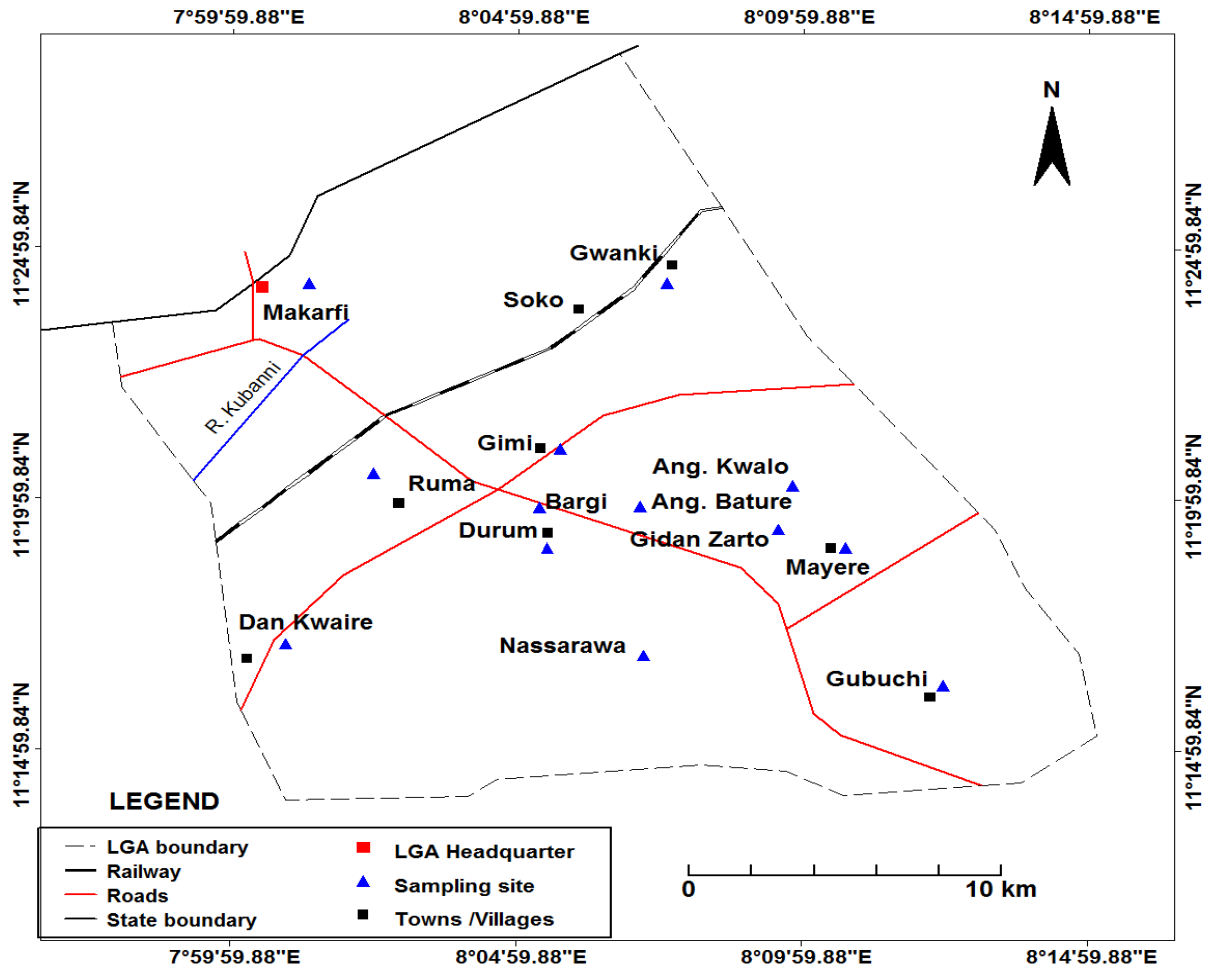


Figure 1. Map showing location of sampling areas. Source: Modified from administrative map of Kaduna State.

least was the Fararkwama (18% disease incidence).

Identification of the virus isolate involve in SCMD in Makarfi Local Government

Enzyme-linked immunosorbent assay (ELISA) analysis of samples obtained showed that SCMV was the most common type detected (Table 1) followed by SrMV and MDMV (Table 2). Out of the 63 samples tested, 52 (83%) samples reacted positive to SCMV antiserum, six (0.1%) were positive to MDMV and three (0.01%) positive to SrMV antisera. JGMV was not detected in the samples obtained from the 14 villages. This showed that SCMV have the highest occurrence. SCMV was detected in 49 purple cane and in three white cane fields from all the fourteen villages visited, while SrMV was detected in 3 (2 white canes and 1 purple cane) from Ruma and 3 (all of the purple cane) from Makarfi town and MDMV was detected in (one white cane) from Bargi and (2 purple cane) from Makarfi town.

However, mixed infections of SCMV and SrMV were recorded in two white canes from Ruma and one purple cane from Makarfi village. Mixed infections of the three viruses: SCMV, MDMV and SrMV were also recorded in two purple land races in the field from Makarfi village (Table 3). A range of ELISA values for the 63 survey samples, healthy controls and negative controls for the four different viruses inducing Sugarcane Mosaic Disease are shown in (Table 4). The mean values for SCMV, JGMV, MDMV and SrMV positive controls were 3.378, 3.317, 3.535 and 3.116 nm while their negative controls were 1.256, 1.190, 1.024 and 0.779 nm, respectively. The absorbance values show that SCMV has the highest concentration followed by MDMV and SrMV.

DISCUSSION

Makarfi has a long history of sugarcane cultivation, the weather and soil conditions support the extensive cultivation of the crop. As such there is a popular saying that Makarfi is the home of sugarcane. Three land races

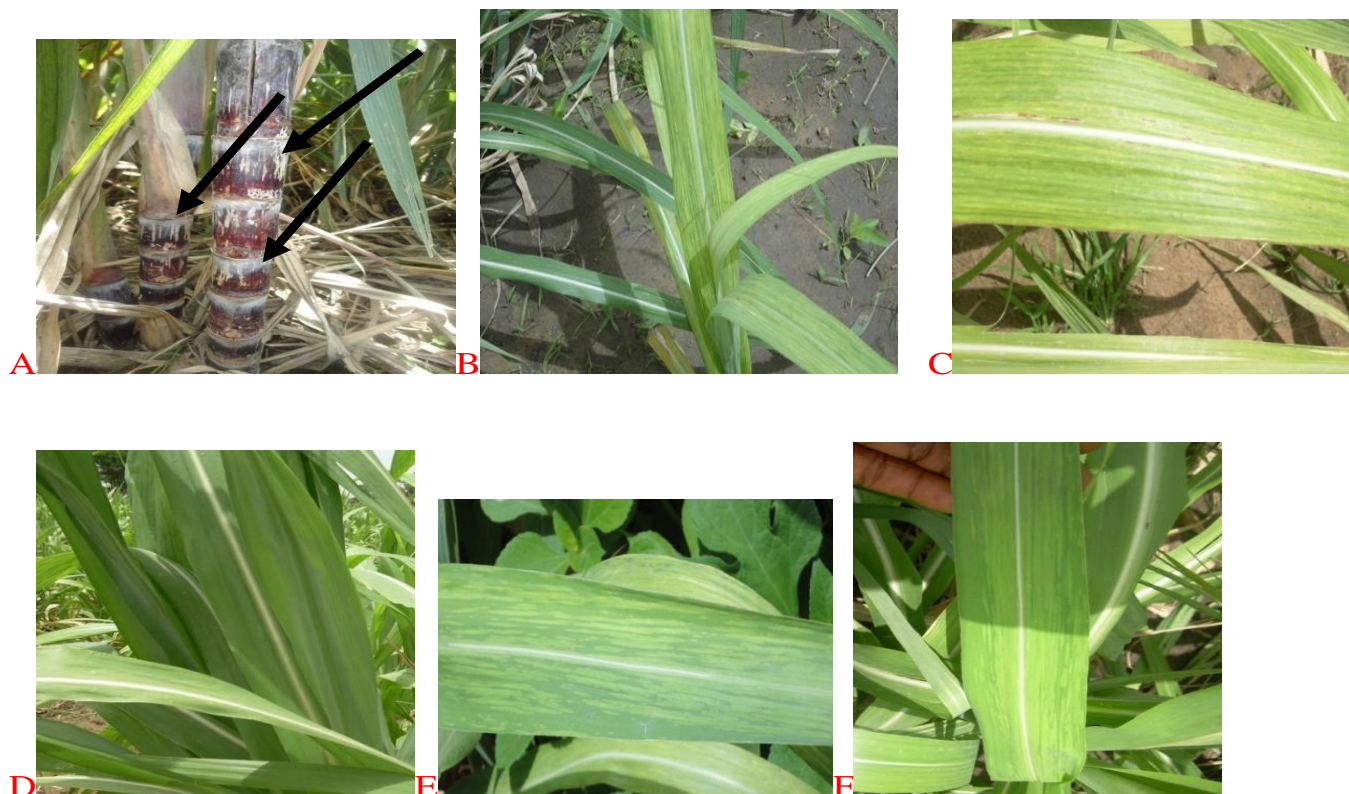


Figure 2. A- White Stripe and short internodes symptoms on infected stem. B, C- Yellow chlorotic stripes. D- Healthy leaves. E, F- Pale green stripes on sugarcane leaves.

Table 1. Numbers of DAS-ELISA positive samples from symptomatic and asymptomatic cultivated sugarcane leaves samples from Makarfi local government areas.

Location	Land race	Number of samples tested	Virus detected	
			SCMV	JGMV
Mayere	purple cane	4	4	0
Ruma	purple cane	6	5	0
	green cane	2	2	0
Nassarawa	purple cane	1	1	0
	white cane	1	0	0
Gimi	purple cane	2	2	0
GidanZarto	purple cane	5	5	0
Ang.kwalo	purple cane	4	3	0
Dankwaire	purple cane	4	3	0
Makarfi	purple cane	8	7	0
Durum	white cane	2	1	0
Durum	purple cane	5	3	0
Gubuchi	purple cane	5	4	0
Bargi	purple cane	3	3	0
	white cane	2	1	0
Gwanki	purple cane	5	5	0
Ang.Bature	purple cane	4	3	0
Total		63	52	0

Table 2. Numbers of TAS-ELISA positive samples from symptomatic and asymptomatic cultivated sugarcane leaves samples from Makarfi Local Government areas.

Location	Land race	Number of samples tested	Virus detected	
			MDMV	SrMV
Mayere	purple cane	4	0	0
Ruma	purple cane	6	0	1
	Green cane	2	0	2
Nassarawa	purple cane	1	0	0
	white cane	1	0	0
Gimi	purple cane	2	0	0
GidanZarto	purple cane	5	0	0
Ang.kwalo	purple cane	4	0	0
Dankwaire	purple cane	4	0	0
Makarfi	purple cane	8	2	3
Durum	white cane	2	0	0
Durum	purple cane	5	0	0
Gubuchi	purple cane	5	0	0
Bargi	purple cane	3	0	0
	white cane	2	1	0
Gwanki	purple cane	5	0	0
Ang.Bature	purple cane	4	0	0
Total		63	3	6

Table 3. Natural occurrence of double and triple infections of sugarcane virus isolates of SCMD in Makarfi L.G.

Location	Land race	Detection of mix infections of	
		SCMV and SrMV	SCMV, MDMV and SrMV
Makarfi	purple cane	1	2
Ruma	white cane	2	0

Table 4. Mean SCMD complex enzyme-linked immunosorbent assay (ELISA) absorbance (A_{405nm}) values for all samples tested.

Antibodies	Positive control	Negative control	Field sample (range)
SCMV	3.378	1.256	1.218-3.717
JGMV	3.317	1.190	0.476-2.091
MDMV	3.535	1.024	0.847-3.125
SrMV	3.116	0.779	0.398-3.106

are grown there, BakarKwama”, “fararKwama” and BaHausa, however the distribution of these cultivars vary within the local government. SCMD has been very devastating in all the areas visited. Initially, the farmers associated the disease to urea fertilizer application but they later confirm that the symptoms manifested even in fields where urea is not applied as such they named it “Mamar” or “Maizabuwa”. The SCMD is the most devastating disease of cane in the areas especially where the

“Bakarkwama” (the most susceptible land race) dominates. However, the farmers were not certain of their source of infection. The source of infection might be in weeds or cereals intercropped with sugarcane for example, *Zea mays* and *Sorghum bicolor*. Xu et al. (2008) reported SCMV and SrMV infections in maize and sorghum in China. Sharma and Misra (2011) reported high incidence of MDMV in China, South Africa and United States of America in maize. *A. gossypii* may be responsi-

ble for the spread but to a larger extent contaminated cutlass may be responsible. Singh et al. (2005) confirmed four species of aphids to transmit SCMV in sugarcane in India. The results of DAS and TAS ELISA indicate the occurrence of the three viruses (SCMV, MDMV and SrMV) causing mosaic of sugarcane and the non detection of JGMV in all the samples tested. Different immunological techniques have been used to distinguish the four SCMD complexes (Tosic, 1990). For example, Yasmin et al. (2011) reported the occurrence of SCMV and MDMV in two provinces of Pakistan while SCMV and SrMV were found to be the causal agents of sugarcane mosaic disease in South China (Xu, 2005). However, Mohammad and Behzad (2009) reported the detection of SCMV but not MDMV and SrMV in Tehran province of Iran. However, in another study using ELISA, Selfer et al. (2005) detected the presence of JGMV from sorghum in Nigeria, where the isolate induces necrosis in sorghum but fail to infect Johnsongrass and oat. The information on SCMD subgroup present in a particular area will be of great economic importance in establishing the losses caused by SCMD in the area.

The results show that SCMV type is the most common of the SCMD viruses as it was detected in all locations visited in Makarfi Local Government Area. SCMV is followed by SrMV and MDMV. The occurrence of SCMV has also been reported in other countries like Huckett et al. (1998) who confirmed the presence of SCMV in South Africa using genome based technique. Also, Saleem et al. (2011) confirmed the presence of SCMV from naturally infected sugarcane crop in Pakistan. Of interest are the detection of the three viruses and the non detection of JGMV in sugarcane. The non detection of JGMV in all the samples tested may be because MDMV, SCMV and SrMV are closely related to each other than they are to JGMV (Shukla et al., 1992, Seifers et al., 2005).

The highest incidence of SCMV suggests its long existence in the areas, the crop situation observed showed that the farmers are using continuously their own germplasm so that virus is accumulating in the field and this is a major factor in disease development and spread. The possible reasons for the high incidence of viral infection may be due to susceptibility of sugarcane varieties, lack of a viral screening system (Zhou and Xu, 2005), high densities of aphid populations, which transmit sugarcane mosaic disease (Luo et al., 2003), and also the presence of viral inoculum reservoirs available near sugarcane-growing areas (Zhou et al., 2007). Samples with mixed infections were also observed, one purple cane from Makarfi and two white canes from Ruma were co-infected with SCMV and SrMV while two other purple canes from Makarfi were co-infected with SCMV, MDMV and SrMV. This is in agreement with findings of Xu et al. (2008) in which a high incidence of SCMV and SrMV co-infection was revealed in both hybrid and noble sugarcane, all co-infected plants showed mosaic symptom. With the introduction of new improved commercial cultivars of sugarcane

and other cereals like maize and sorghum, these triple and double infections might lead to more devastating disease. The detection of SCMV in asymptomatic plants suggests that latent infection occur or a mild strain of the virus.

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Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Protocol optimization for *in vitro* mass propagation of two sugarcane (*Saccharum officinarum* L.) clones grown in Ethiopia

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The present study was initiated to optimize *in vitro* protocol for mass propagation of two commercial sugarcane clones (Co 449 and Co 678) grown in Ethiopia through shoot tip culture. Experiments on shoot multiplication and rooting were laid out in a completely randomized design with factorial treatment arrangements. Shoot tips were surface sterilized with 5% active chlorinated Berekina for 25 min and initiated on Murashige and Skoog (MS) medium supplemented with 2 mg L⁻¹ 6- benzylamino purine + 0.5 mg L⁻¹ indole-3- butyric acid. For *in vitro* multiplication, aseptically initiated shoot tips were treated with different concentrations and combinations of BAP and Kin using either sucrose or table sugar in separate experiments. For root induction, regenerated shoots were transferred onto half MS medium supplied with 6% sucrose or table sugar and different concentrations and combinations of IBA and NAA. With regard to shoot multiplication, genotype Co449 showed maximum regeneration frequency of 80% with 7.87 ± 1.06 shoots per explants on MS medium with 3% sucrose and 2 mg L⁻¹ BAP + 0.25 mg L⁻¹ Kin. On the same carbon source, genotype Co678 showed the highest multiplication frequency of 90% with 9.10 ± 0.10 shoots per explant on medium supplied with 2 mg L⁻¹ BAP + 0.5 mg L⁻¹ Kin. On MS with 4% table sugar and 2 mg L⁻¹ BAP + 0.25 mg L⁻¹ Kin, 80% of the transferred explants of genotype Co449 produced multiple shoots with an average number of 7.61 ± 0.10 shoots per explant while genotype Co678 showed the highest regeneration frequency (86.67%) with mean shoots number per explant of 8.36 ± 0.04 on medium supplemented with 2 mg L⁻¹ BAP + 0.5 mg L⁻¹ Kin. Half MS + 6% sucrose + 2.5 mg L⁻¹ IBA induced the highest rooting (86.67%) with an average root number per shoot of 16.53 ± 0.02 in Co449 cultures. In genotype Co678, ½ MS + 6% sucrose + 5 mg L⁻¹ NAA induced the highest rooting response of 80% with an average root number per shoot of 13.17 ± 0.29. Equivalent rooting responses were recorded on half MS medium supplemented with 6% table sugar. On ½ MS with 6 % table sugar, 2.5 mg L⁻¹ IBA induced the highest rooting response (86.67%) and root number (15.93 ± 0.81) in genotype Co449 while 5 mg L⁻¹ NAA gave the maximum (80%) rooting with average roots per shoot of 13.93 ± 0.81 in genotype Co678. Rooted shoots were transplanted in the green house for hardening and different survival rate was recorded.

Key words: *Saccharum officinarum*, multiplication, rooting, sucrose, table sugar, *in vitro*.

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is a herbaceous perennial crop plant that belongs to the family Poaceae (Singh, 2003; Sharma, 2005; Cha-um et al., 2006). It has chromosome number of $2n = 80$ (Daniels and Roach, 1987; Asano et al., 2004). It had been thought to be evolved in Asia, probably the island of New Guinea (Singh, 2003). Today, sugarcane is cultivated in over 110 countries and 50% of the production occurs in Brazil and India (FAO, 2008). Sugarcane contributes nearly 70% of global annual sugar production (Sengar, 2010).

Sugarcane is one of the most widely grown crops in Ethiopia, even though the history is not well-documented when it was introduced (Assefa, 2006). According to Tafesse and Haile-Michael (2001), the Dutch Company, Handles-Vereenging Amsterdam (HVA) pioneered commercial cultivation of sugarcane in Wanji, Ethiopia in 1954/55. In Ethiopia, sugarcane cultivation has multipurpose. The sugar juice is used for making sugar. Molasses (thick syrupy residue) is used in the production of ethanol (blended for motor fuel) and as livestock feed. The bagasse (fibrous portion) is burned to provide heat and electricity for sugar mills and green tops can be used as cattle feed. Furthermore, sugar factories being located in rural areas, they generate employment opportunity for thousands of people at various stages of production (Girma and Awulachew, 2007). At the moment, there are three large scale sugar establishments in Ethiopia: Wonji/Shoa, Matahara and Fincha, producing a total of about 300,000 tons of sugar and eight million liter of ethanol per year (Feyissa et al., 2010). However, the current production is not satisfying the existing domestic sugar and its by-products demand. As a result, establishment of 10 new sugar factories and expansion of the existing ones are underway with the aim of producing 2.5 million tons sugar and 304 million liter ethanol at the end of the growth and transformation plan (GTP) (Ambachew and Firehun, 2010). Currently, sugarcane is cultivated on about 33,777 ha of land in the country, of which 30,157 ha is government owned and the remaining portion is private owned. It is planned to increase the government owned sugarcane plantation to 200,000 ha at the end of the Ethiopian growth and transformation plan (Feyissa et al., 2010). The increase in plantation area creates high demand of good planting material to be available and these calls for a means that can provide planting material in large scale and within short period of time.

However, the current conventional seed cane production method where stem cuttings with two or three nodes used as planting material, has various limitations. The seed multiplication rate is too low (1:6 to 1:8) which makes the spread of newly released varieties slow, taking

over 10 years to scale up a newly released variety to the commercial level (Cheema and Hussain, 2004; Sengar, 2010), and also facilitates the spread of pathogens and may result in epidemics (Schenck and Lehrer, 2000). Moreover, the method requires large nursery space (Sundara, 2000). Therefore, developing an efficient propagation system for mass multiplication of sterile sugarcane planting material of selected variety is of paramount importance. In line with this, application of micropropagation techniques for the propagation of sugarcane has the benefits of rapid propagation of new cane varieties, reduction in seed use, regeneration of large number of true to type plantlets from a small tissue, elimination of pathogens and storage of plant germ-plasm under aseptic condition (Ali et al., 2004; Gosal et al., 2006; Khan et al., 2006). However, implementation of micropropagation technology is influenced by many factors such as production cost and knowhow of micropropagation protocols, which make the technology expensive and unaffordable by less developed countries (Demo et al., 2008).

In plant culture, no two genotypes give similar response under a given set of culture conditions (Nehara et al., 1989; 1990a). It often requires testing of various type, concentration and mixture of the growth regulators during the development of a tissue culture protocol for a new plant tissue (Bhojwani and Razdan, 1996). With this reason, standardization of protocols for *in vitro* multiplication of sugarcane through callus culture, axillary bud and shoot tip culture have been reported by many authors (Barba et al., 1978; Bakesha et al., 2002; Alam et al., 2003; Ali et al., 2008; Behara and Sahoo, 2009; Khan et al., 2009).

So far, there is no report that is adopted for *in vitro* mass propagation of sugarcane genotypes grown in Ethiopia, and due to this the country is not getting advantage of this modern technology. Therefore, the present study was conducted to develop/optimize *in vitro* protocol for mass propagation of two sugarcane clones (Co 449 and Co 678) grown in Ethiopia through shoot tip culture.

MATERIALS AND METHODS

The study was conducted at plant tissue culture laboratory of Jimma University College of Agriculture and Veterinary Medicine, Ethiopia. Two sugarcane genotypes, Co449 and Co678, were considered in this study. They were obtained from Matahara Sugar Estate under the license of Ethiopian Sugar Corporation. To reduce explants sourced contamination, the stock plants were raised by planting seed canes under greenhouse condition. For *in vitro* studies, shoot tips were excised from tops of three to four months-old actively growing sugarcane raised in the greenhouse. The

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Table 1. ANOVA summary of effect of BAP and Kin on shoot multiplication and shoot growth on MS with sucrose.

Source of variation	DF	Mean square			
		Multiplication rate	Number of shoots/explant	Shoot length	Number of leaves per hoot
Gen	1	56.02***	2.74***	271.85***	3.14***
Kin	2	3672.28***	56.81***	1491.82***	12.81**
BAP	4	13840.35***	90.19***	2079.67***	36.17***
Gen *Kin	2	199.53***	1.23***	38.50***	0.86***
Gen *BAP	4	190.72***	0.35*	18.91***	0.49***
Kin *BAP	8	51.54***	1.20***	158.63***	0.56***
Gen *Kin *BAP	8	149.19***	1.48***	6.60***	0.25***
CV (%)		5.48	8.66	3.04	7.23

***= Very highly significant ($P < 0.0001$) at $\alpha = 0.05$ significance level, *= significant ($P = 0.03$) at $\alpha = 0.05$ significance level, DF= Degree of freedom, Gen = Sugarcane genotypes, Kin=Kinetin and BAP =6-Benzylaminopurine

leaves were removed and the shoot blocks were taken to the laboratory. In the laboratory, surrounding leaf sheaths were carefully removed one by one until the inner white sheaths were exposed. Then, 10 cm long tops were collected by cutting off at the two ends, locating the growing point somewhere in the middle of the top. The shoot tip blocks were washed under running tap water for 30 min with soap solution which was followed by treating with 0.3% Kocide (fungicide solution) for one and half hour under laminar air flow. After decanting the kocide, shoot tip blocks were rinsed three times with sterile distilled water and then treated with 70% ethanol for 30 s. Then, after three times rinsing with sterile distilled water, the explants were treated with Berekina (with 5% active ingredient of chlorine) for 25 min. To increase efficacy, two drops of Tween-20 solution was added into Berekina solution. Decanting the sterilizing solution under safe condition, the explants were washed three times each for 5 min with sterile distilled water and were left for 10 min to make their surface dry. Then, leaf sheaths damaged during sterilization were removed by using sterilized forceps. Finally, 2 cm long shoot tips were excised with sterilized scarplines and cultured on MS basal medium supplemented with 2 mg L⁻¹ BAP + 0.5 mg L⁻¹ IBA, 3% sucrose, 0.8% agar (Bakasha et al., 2002). For shoot multiplication, aseptically initiated 3 cm long cultures were transferred to MS basal medium supplemented with 3% sucrose or 4% table sugar, 0.8% agar and varying concentrations of BAP (0.0, 0.5, 1.0, 1.5 and 2.0 mg L⁻¹) and Kin (0.0, 0.25 and 0.5 mg L⁻¹) in factorial combination. To avoid the carry over effect of multiplication media on *in vitro* rooting, multiplied shoots were maintained on plant growth regulators free MS basal medium for the next two weeks. The rooting response of *in vitro* regenerated shoots was considered on half strength MS basal medium supplemented with 6% sucrose or 6% table sugar, 0.8% agar and different concentration of IBA (0.0, 1.25, 2.5, 3.75 and 5 mg L⁻¹) in factorial combination with NAA (0.0, 1.25, 2.5, 3.75 and 5 mg L⁻¹). In all the cases, 15 explants were cultured per treatment combination and cultures were maintained in growth chamber conditions of temperature of 25 ± 2°C, 16 h light photoperiod, relative humidity of 70-80%, and fluorescent light intensity of 2500 lux. Plantlets with well developed shoots and roots were transplanted in plastic pot containing a mixture of sieved river sand, forest soil and well decomposed farm yard manure (FYM) in a 1:1:1 ratio and transferred to greenhouse for hardening. The transplanted plantlets were kept in greenhouse under shade of polyethylene sheets for 14 days and were sprayed with water two to three times every day in the first month and once in the remaining time. After 45 days, observation on percentage of plantlets that were successfully acclimatized was recorded. For the multiplication experiment, percent of regenerated explants, average number of shoots per explant, average shoots length (cm) and average number of leaves per shoots were recorded for each treatment

combination after 30 days of transfer. Regarding root regeneration experiment, percentage of microshoots rooted, average number of roots per microshoot, and average root length (cm) were recorded after 30 days of culture transfer from plant growth regulators (PGR) free MS medium on to root induction medium. All collected data were subjected to three way ANOVA using SAS software version 9.2 (SAS Institute Inc., 2008). In all the cases, statistical significance was computed at $\alpha = 5\%$ and treatment mean separation was done using procedure of REGWQ (Ryan, Elinot, Gabriel, and Welsh) multiple range test.

RESULTS AND DISCUSSION

Effect of BAP and Kin on shoot multiplication of sugarcane *in vitro* cultures on MS medium with sucrose

ANOVA showed that genotype, kinetin, BAP and their interactions had very high significant ($p < 0.0001$) effects on shoot multiplication frequency, average shoots number, average shoot length and average number of leaves per shoot (Table 1). Interaction of genotype (Gen), Kinetin (Kin) and BAP (Gen*Kin*BAP) revealed that all the three factors are dependent on each other for *in vitro* multiplication of sugarcane.

For both genotypes, the lowest multiplication response (0%) was recorded on MS basal medium devoid of BAP and Kin while multiple shoot formation occurred in the presence of BAP and Kin (Table 2). The use of 2 mg L⁻¹ BAP without Kin produced 4.56 shoots per explants in genotype Co449, which was significantly improved to 7.87 shoots by addition of 0.25 mg L⁻¹ Kin (Table 2). Similar trend of increase in number of shoots per explants was observed for genotype Co678 with an inclusion of Kin. These showed the importance of including Kinetin along with BAP in shoot multiplication media. This essentially indicates that the use of cytokinins (BAP and Kin) have a positive effects and play important role in multiplication of sugarcane cultures. In fact, cytokinins (BAP and Kin) stimulate protein synthesis and participate in cell cycle control and if added into shoot culture media,

Table 2. Effect of BAP and Kin on *in vitro* shoot multiplication of sugarcane cultures on MS with sucrose.

Genotype	Hormone combination		Percentage of Explants multiplied	Number of Shoots/Explant (Mean ± SD)	Shoot length (cm) (Mean ± SD)	Number of Leaves/Shoot (Mean ± SD)
	Kin (mg/L)	BAP (mg/L)				
Co449	0	0	0.00 ^o	0.00 ⁿ ± 0.00	0.00 ^k ± 0.15	0.00 ^s ± 0.00
	0	0.5	6.67 ⁿ	1.78 ^{klm} ± 0.77	3.40 ^{ij} ± 0.15	2.22 ^{opq} ± 0.19
	0	1	20.00 ^l	2.67 ^{ijk} ± 0.58	4.10 ^{ij} ± 0.10	3.00 ^{lmn} ± 0.00
	0	1.5	46.67 ^h	3.51 ^{ih} ± 0.16	5.10 ^{hg} ± 0.06	3.32 ^{j-m} ± 0.34
	0	2	73.33 ^d	4.56 ^{efg} ± 0.38	6.20 ^{bc} ± 0.20	4.67 ^{b-e} ± 0.00
	0.25	0	6.67 ⁿ	0.33 ⁿ ± 0.58	3.13 ^j ± 0.06	1.57 ^{qr} ± 0.12
	0.25	0.5	26.67 ^k	2.33 ^{ljk} ± 0.00	4.27 ^j ± 0.12	2.87 ^{mno} ± 0.12
	0.25	1	40.00 ^j	2.88 ^{ij} ± 0.51	5.23 ^{efg} ± 0.06	3.44 ^{i-m} ± 0.51
	0.25	1.5	66.66 ^e	5.16 ^{def} ± 0.12	6.40 ^{def} ± 0.10	4.10 ^{d-i} ± 0.17
	0.25	2	80.00 ^c	7.87 ^b ± 1.06	6.33 ^{bc} ± 0.21	5.44 ^a ± 0.19
	0.5	0	13.33 ^m	1.66 ^{lm} ± 0.58	4.30 ^{ij} ± 0.10	1.50 ^f ± 0.00
	0.5	0.5	20.00 ^l	4.11 ^{gh} ± 0.19	5.43 ^{def} ± 0.15	2.83 ^{mno} ± 0.29
	0.5	1	46.67 ^h	5.44 ^{dce} ± 0.19	5.90 ^{def} ± 0.10	3.89 ^{fj} ± 0.29
	0.5	1.5	73.00 ^d	6.23 ^c ± 0.08	6.53 ^{cde} ± 0.06	4.75 ^{bcd} ± 0.21
	0.5	2	80.00 ^c	7.33 ^b ± 0.03	6.37 ^{ab} ± 0.25	5.03 ^{abc} ± 0.15
Co678	0	0	0.00 ^o	0.00 ⁿ ± 0.00	0.00 ^k ± 0.00	0.00 ^s ± 0.00
	0	0.5	13.33 ^m	1.56 ^{lm} ± 0.19	3.47 ^{ij} ± 0.06	2.27 ^{op} ± 0.06
	0	1	26.67 ^k	2.11 ^{j-m} ± 0.38	4.27 ^{hg} ± 0.15	3.20 ^{k-n} ± 0.17
	0	1.5	40.00 ^j	2.67 ^{ijk} ± 0.58	5.30 ^{def} ± 0.26	4.00 ^{e-j} ± 0.00
	0	2	60.00 ^f	5.63 ^{dc} ± 0.04	6.23 ^{abc} ± 0.25	4.33 ^{c-f} ± 0.58
	0.25	0	13.33 ^m	1.33 ^m ± 0.00	3.07 ^{ij} ± 0.12	2.03 ^{pqr} ± 0.58
	0.25	0.5	20.00 ^l	3.56 ^{ih} ± 0.69	3.77 ^{hi} ± 0.06	3.54 ^{h-m} ± 0.21
	0.25	1	33.33 ^j	4.44 ^{gf} ± 0.19	5.33 ^{def} ± 0.15	4.22 ^{d-h} ± 0.37
	0.25	1.5	60.00 ^f	5.39 ^{dce} ± 0.10	6.50 ^{ab} ± 0.61	4.31 ^{d-g} ± 0.30
	0.25	2	86.00 ^b	7.21 ^b ± 0.02	6.93 ^a ± 0.12	4.53 ^{b-f} ± 0.50
	0.5	0	20.00 ^l	2.22 ^{j-m} ± 0.19	3.13 ^{ij} ± 0.06	2.54 ^{nop} ± 0.21
	0.5	0.5	46.67 ^h	3.36 ^{ih} ± 0.69	4.27 ^{hg} ± 0.12	3.67 ^{g-l} ± 0.00
	0.5	1	53.33 ^g	5.42 ^{dce} ± 0.21	5.67 ^{cd} ± 0.21	4.70 ^{b-e} ± 0.26
	0.5	1.5	60.00 ^f	7.11 ^b ± 0.13	6.63 ^{ab} ± 0.06	5.17 ^{ab} ± 0.29
	0.5	2	90.00 ^a	9.10 ^a ± 0.10	6.83 ^a ± 0.12	5.67 ^a ± 0.00
CV (%)			5.48	8.66	3.04	7.24

Means in column with the same letter are not significantly different by Ryan-Einot-Gabriel-Welsch Multiple Range Test at $\alpha = 5\%$ significant level.

stimulate lateral bud growth and thus causing multiple shoot formation by breaking shoot apical dominance (Trigiano and Gray, 2005; George and Klerk, 2008).

Among the various concentrations and combinations, MS medium supplemented with 2 mg L⁻¹ BAP + 0.25 mg L⁻¹ Kin and 2 mg L⁻¹ BAP + 0.5 mg L⁻¹ Kin showed maximum shoot multiplication frequency (80%) in sugarcane genotype Co449 (Table 2). On MS medium + 2 mg L⁻¹ BAP + 0.25 mg L⁻¹ Kin, genotype Co449 gave an average of 7.87 ± 1.06 shoot per explant with shoot length of 6.33 ± 0.21 cm and leaf number of 5.44 ± 0.19 per shoot; raising concentration of Kin to 0.5 mg L⁻¹ on the same media composition for Co449 did not result in better number of shoots per explants.

On the other hand, genotype Co678 showed the highest shoot multiplication on MS medium supplemented with 2 mg L⁻¹ BAP + 0.5 mg L⁻¹ Kin where 90% of the transferred cultures showed multiple shooting with an average of 9.10 ± 0.10 shoots per culture, 6.83 ± 0.12 cm shoot length and 5.67 ± 0.00 leaves per shoot. MS + 2 mg L⁻¹ BAP + 0.25 mg L⁻¹ Kin, which showed the highest number of shoots per explants in genotype Co449, resulted in 7.21 shoots per explants with 86% of the explants showing multiple shoot formation for Co678 and thus this medium can be considered as the second best medium combination for this genotype.

The current result is consistent with other *in vitro* multiplication reports of sugarcane using 3% sucrose in MS

Table 3. ANOVA summary of effect of BAP and Kin on shoot multiplication and shoot growth on MS with table sugar.

Source of variation	DF	Mean square			
		Multiplication rate	Number of shoots/explant	Shoot length	Number of leaves / shoot
Gen	1	18.67***	3.67** *	6.51***	3.90***
Kin	2	2794.71***	49.18***	35.17***	11.97***
BAP	4	11836.35***	78.08***	48.25***	36.67***
Gen * Kin	2	454.69***	0.30**	0.17***	0.67***
Gen *BAP	4	118.11***	0.96***	0.44***	0.57***
Kin * BAP	8	87.48***	1.35***	3.58***	0.69***
Gen * Kin *BAP	8	72.49***	1.46***	0.16***	0.28***
CV (%)		5.28	5.77	3.03	6.81

***= Very highly significant ($P < 0.0001$) at $\alpha=0.05$ significance level, ** = highly significant ($P = 0.0044$) at $\alpha=0.05$ significance level, DF= Degree of freedom, Gen = Sugarcane genotypes, Kin=Kinetin and BAP =6-Benzylaminopurine.

medium. Khan et al. (2009) found maximum (7) shoot number per explants with 8.5 cm shoot length on MS medium supplemented with 1.0 mg L⁻¹ BAP and 0.5 mg L⁻¹ Kin in sugarcane variety CP-77-400. In addition, they observed 6 shoots per explants on MS medium with 1.0 mg L⁻¹ BAP + 0.1 mg L⁻¹ Kin in sugarcane variety CPF-237 and 8 shoots per explants on MS media supplemented with 1.0 mg L⁻¹ BAP + 0.5 Kin in sugarcane variety HSF-240. In the present study, an average shoot number of 5.44 in genotype Co449 and 5.42 shoots in genotype Co678 were observed on MS medium with 1.0 mg L⁻¹ BAP + 0.5 mg L⁻¹ Kin which are almost in line with the report of Khan et al. (2009) at this level. Singh (2003) observed an average of 12.33 shoots per explants on MS medium supplemented with 1.5 mg L⁻¹ BAP and 0.5 mg L⁻¹ Kin. Bakesha et al. (2002) reported multiple shoots from shoot tip explants of sugarcane cultured on MS medium supplemented with BAP (0.5 - 2.0 mg L⁻¹) and Kin (0.5 mg L⁻¹). In terms of shooting frequency, the present result agree with the report of Behera and Sahoo (2009) and Biradar et al. (2009) who respectively reported 92% and 79.64% shoot multiplication frequency in sugarcane *in vitro* micropropagation. Therefore, 2 mg L⁻¹ BAP + 0.25 mg L⁻¹ Kin is the optimum and best hormones combination for maximum *in vitro* shoot multiplication of sugarcane genotype Co449 cultures. While MS medium supplemented with 2 mg L⁻¹BAP + 0.5 mg L⁻¹Kin is the best combination for *in vitro* shoot multiplication of sugarcane genotype Co678 cultures.

Effect of BAP and Kin on shoot multiplication of sugarcane *in vitro* cultures on MS medium with table sugar

ANOVA result showed that all the main and interaction effects of genotype, Kin and BAP were very highly significant ($p < 0.0001$) on percentage of shoot induction, average number of shoots per-explants, shoot length and leaves number per shoot (Table 3). Interaction among genotype, Kin and BAP (Gen*Kin*BAP = $p < 0.0001$) showed interdependence of the three factors for shoot

multiplication in the presence of table sugar. The multiplication difference between the two genotypes might be contributed by their difference in level of endogenously accumulated auxins and cytokinins (George and Klerk, 2008).

Among different combinations of BAP and Kin, genotype Co449 showed the highest multiple shoot formation and growth on MS medium supplemented with BAP (2.0 mg L⁻¹) + Kin (0.25 mg L⁻¹) (Table 4) where 80% of the transferred explants produced multiple shoots (7.61 ± 0.10 shoots per explants) that are on an average 6.40 ± 0.10 cm long with 5.33 ± 0.00 leaves per shoot. Increasing concentration of Kin from 0.25 mg L⁻¹ to 0.5 mg L⁻¹ under the same media composition for the same genotype (Co449) reduced frequency of shoot formation to 73.33% and number of shoots per explant to 6.81. On the other hand, genotype Co678 showed maximum multiplication response on MS medium supplemented with 2 mg L⁻¹BAP + 0.5 mg L⁻¹ Kin where 86.67% of the transferred explants gave multiple shoot formation with 8.36 ± 0.04 shoots per explants, 7.27 ± 0.11 cm mean shoot length and 5.56 ± 0.33 leaves per shoot. When lower concentration of Kin (0.25 mg L⁻¹) was used for the same genotype (Co678) on the same media composition, reduced frequency of multiple shoot formation (73.33%) and fewer shoots per explants (7.26 ± 0.13) were achieved.

On the contrary, in both genotypes, the lowest multiplication response (0%) was observed on MS medium devoid of BAP and Kin while shoot multiplication was observed in media containing BAP and Kin (Table 4). This clearly indicates the significance of adding BAP and Kin in tissue culture media for shoot multiplication of sugarcane cultures. Indeed, cytokinins (BAP and Kin) enhance multiple shoots induction by overcoming apical dominance and releasing lateral buds from dormancy which results in shoot proliferation (Trigiano and Gray, 2005; George and Klerk, 2008). It was also observed that hormone combination that resulted in maximum shoots per explants responded relatively shorter shoot length. This might be the effect of high level of cytokinins that inhibited shoot elongation (George and Klerk, 2008).

Table 4. Effect of BAP and Kin on *in vitro* shoot multiplication of sugarcane cultures on MS medium with table sugar.

Genotype	Hormone combination		Percentage of explants multiplied	Number of Shoots/Explant (Mean ± sd)	Shoot length (cm) (Mean ± SD)	Number of Leaves/Shoot (Mean ± SD)
	Kin (mg/L)	BAP (mg/L)				
Co449	0	0	0.00 ⁿ	0.00 ^l ± 0.00	0.00 ^h ± 0.15	0.00 ⁿ ± 0.00
	0	0.5	13.33 ^l	1.33 ^j ± 0.00	3.47 ^g ± 0.15	2.33 ^{kl} ± 0.00
	0	1	20.00 ^k	2.31 ⁱ ± 0.02	4.10 ^f ± 0.10	3.11 ^{hij} ± 0.19
	0	1.5	53.33 ^f	4.21 ^f ± 0.11	5.17 ^d ± 0.06	3.43 ^{ghi} ± 0.20
	0	2	73.33 ^c	4.36 ^f ± 0.04	6.20 ^{cb} ± 0.20	4.78 ^{bc} ± 0.19
	0.25	0	6.67 ^m	0.67 ^k ± 0.58	3.13 ^g ± 0.06	1.62 ^m ± 0.11
	0.25	0.5	26.67 ^j	2.57 ⁱ ± 0.06	4.27 ^{ef} ± 0.12	2.89 ^{ijk} ± 0.19
	0.25	1	40.00 ^h	2.73 ^{hi} ± 0.12	5.23 ^d ± 0.06	3.56 ^{f-i} ± 0.39
	0.25	1.5	66.67 ^d	5.40 ^e ± 0.10	6.33 ^b ± 0.21	4.21 ^{c-f} ± 0.18
	0.25	2	80.00 ^b	7.61 ^b ± 0.10	6.40 ^b ± 0.10	5.33 ^{ab} ± 0.00
	0.5	0	20.00 ^k	2.41 ⁱ ± 0.17	4.30 ^{ef} ± 0.10	1.56 ^m ± 0.02
	0.5	0.5	26.67 ^j	3.23 ^{gh} ± 0.12	5.43 ^d ± 0.15	2.93 ^{ijk} ± 0.12
	0.5	1	46.67 ^g	5.36 ^e ± 0.04	5.90 ^c ± 0.12	3.83 ^{d-g} ± 0.29
	0.5	1.5	60.00 ^e	6.38 ^d ± 0.04	6.53 ^b ± 0.06	4.86 ^{bc} ± 0.23
0.5	2	73.33 ^c	6.81 ^{cd} ± 0.69	6.37 ^b ± 0.25	5.14 ^{ab} ± 0.22	
Co678	0	0	0.00 ⁿ	0.00 ^l ± 0.00	0.00 ^h ± 0.00	0.00 ⁿ ± 0.00
	0	0.5	13.33 ^l	1.71 ^j ± 0.00	3.27 ^g ± 0.06	2.27 ^{kl} ± 0.58
	0	1	26.67 ^j	2.44 ⁱ ± 0.00	4.30 ^{ef} ± 0.26	3.42 ^{ghi} ± 0.21
	0	1.5	40.00 ^h	3.39 ^g ± 0.10	5.13 ^d ± 0.06	4.33 ^{cde} ± 0.36
	0	2	60.00 ^e	5.56 ^e ± 0.19	7.20 ^a ± 0.10	4.44 ^{cd} ± 0.51
	0.25	0	6.67 ^m	2.39 ⁱ ± 0.00	3.20 ^g ± 0.10	2.14 ^{lm} ± 0.17
	0.25	0.5	20.00 ^k	3.78 ^{fg} ± 0.50	5.30 ^d ± 0.36	3.67 ^{e-h} ± 0.00
	0.25	1	33.33 ⁱ	4.11 ^f ± 0.08	6.57 ^b ± 0.12	4.42 ^{cd} ± 0.15
	0.25	1.5	53.33 ^f	4.24 ^f ± 0.77	7.30 ^a ± 0.10	4.44 ^{cd} ± 0.39
	0.25	2	73.33 ^c	7.26 ^{bc} ± 0.13	7.50 ^a ± 0.20	4.67 ^{bc} ± 0.58
	0.5	0	20.00 ^k	2.44 ⁱ ± 0.19	4.60 ^e ± 0.10	2.67 ^{ijkl} ± 0.00
	0.5	0.5	46.67 ^g	3.33 ^g ± 0.00	5.87 ^c ± 0.12	3.77 ^{d-h} ± 0.19
	0.5	1	53.33 ^f	5.17 ^e ± 0.12	6.27 ^{cb} ± 0.35	4.87 ^{bc} ± 0.23
	0.5	1.5	60.00 ^e	7.18 ^{bc} ± 0.14	7.20 ^a ± 0.10	5.17 ^{ab} ± 0.29
0.5	2	86.67 ^a	8.36 ^a ± 0.04	7.27 ^a ± 0.11	5.56 ^a ± 0.33	
CV (%)			5.28	5.76	3.03	6.81

Means in column with the same letter are not significantly different by Ryan-Einot-Gabriel-Welsch Multiple Range Test $\alpha = 0.05$ significance level.

Similar synergetic effect of BAP and Kin combination on shoot multiplication of sugarcane explants was reported by previous studies. Madhulatha et al. (2004) reported that BAP and Kin are generally known to reduce the apical meristem dominance and induce both axillary and adventitious shoots formation from meristematic explants. Geetha and Padmanadhan (2001) reported that the combination of BAP with Kin gave the maximum shoot multiplication response in most sugarcane varieties. Cheema and Hussan (2004) also used the combination of BAP and Kin for the multiplication of six sugar-cane varieties: HSF-240, SPF-213, SPF-234, CP43/33, CP77/400 and CPF237. Ali et al. (2008) observed maximum shoot multiplication response in sugarcane variety BL-4 using the

combination of BAP and Kin in MS medium. Khan et al. (2009) observed maximum (6-11) shoots per explants in MS medium supplemented with BAP (0.0 - 1.5 mg L⁻¹) and Kin (0.0 - 0.5 mg L⁻¹) in three sugarcane varieties. Khan et al. (2006) reported 8.25 ± 0.95 to 11.00 ± 0.81 microshoots per explants using 4% commercial sugar as carbon source in MS media supplemented with BAP.

Hence, on MS medium supplemented with 4% table sugar, BAP (2 mg L⁻¹) + Kin (0.25 mg L⁻¹) was found to be the best combination for shoots multiplication of sugarcane Co449 cultures. While, BAP (2 mg L⁻¹) + Kin (0.5 mg L⁻¹) hormones combination was the best for *in vitro* multiplication of sugarcane genotype Co678 cultures.

The current result indicated that both types of carbon

Table 5. ANOVA summary of effect of IBA and NAA on *in vitro* rooting and root growth on half MS with sucrose.

Source of variation	DF	Mean square		
		Rooting rate	Number of roots/shoot	Root length
Gen	1	96.00***	1.53***	0.29 ***
IBA	4	2357.33***	71.78***	21.75***
NAA	4	2790.68***	20.41***	0.72***
Gen * IBA	4	142.67***	7.02***	0.36***
Gen *NAA	4	302.67***	1.91***	0.23***
IBA * NAA	16	3894.00***	84.42***	22.79***
Gen * IBA *NAA	16	82.67***	2.14***	0.12***
CV (%)		6.89	5.86	5.28

***= Very highly significant ($P \leq 0.0001$) at $\alpha=0.05$ level, DF= Degree of freedom, Gen = Sugarcane genotypes, IBA=Indole-3-butyric acid and NAA= α -naphthaleneacetic acid

source (sucrose or table sugar) showed similar number of shoots per explants and shoot growth comparing Table 2 and 4. Thus, it is possible to deduce that irrespective of the type of carbon source used in the MS medium, 2 mg L⁻¹ BAP + 0.25 mg L⁻¹ Kin is the optimum hormones combination for multiplication of Co449 cultures and 2 mg L⁻¹ BAP + 0.5 mg L⁻¹ Kin is the optimum combination for shoot multiplication of genotype Co678 cultures. This result is consistent with the study by Demo et al. (2008) who observed equivalent potato cultures regeneration on MS medium supplemented with table sugar and graded sucrose. The same authors also reported that table sugar not only enhanced micro-propagation but also significantly lowered the production input costs by 34 to 51% when compared with the analytical grade sucrose. Gamburg (2002) and Kodym and Zapata (2001) also reported superior performances of *in vitro* plantlets of banana, chrysanthemum, peanut, and chickpea in medium supplemented with carbohydrates such as glucose, maltose, and table sugar.

Therefore, the present result proved the possibility of utilizing the locally available (in each shop and supermarket), relatively cheap (currently USD 1-1.5 per kg) table sugar as carbon source in place of graded sucrose which is imported and expensive (USD 147 per kg) product in sugarcane tissue culture. Hence, to make developing countries like Ethiopia beneficiary of micropropagation technology, the utilization of such locally available and economically reasonable resources in place of the expensive ones is best alternative.

Effect of IBA and NAA on rooting of *in vitro* raised sugarcane plantlets on half strength MS with sucrose

ANOVA showed very highly significant ($p < 0.0001$) effect of all main and interaction effect of genotype, IBA and NAA (Table 5) on rooting frequency, average roots number per shoot and root length in both genotypes indicating the interdependence of these factors on *in vitro* root induction of sugarcane *in vitro* shoots. The very highly

significant effect of genotype indicates existence of genotype difference between the two genotypes on their rooting potential for the same level of IBA and NAA combination: indeed genotype Co449 responded higher rooting frequency than genotype Co678 cultures (Table 6).

Rooting response was not observed in both genotypes on half MS medium devoid of IBA and NAA (Table 6). It was also observed that an increase of IBA from 0.0 to 5 mg L⁻¹ maintaining the concentration of NAA at 0.0 mg L⁻¹, increased the rooting frequency of Co449 to 66.67%, with the average roots number per shoot to 7.20 ± 0.10 and average root length to 3.07 ± 0.31 cm. The same trend increased rooting frequency of Co678 cultures to 60%, average roots number per culture to 8.30 ± 0.10 and average roots length to 2.53 ± 0.15 cm. In the absence of IBA, an increase of the concentration of NAA from 0.0 mg L⁻¹ to 5 mg L⁻¹, increased the rooting frequency in both genotype to 80%, an average roots number per shoot and root length respectively to 12.20 ± 0.52 and 7.10 ± 0.57 cm in Co449 and to 13.17 ± 0.29 and 7.63 ± 0.05 cm in sugarcane genotype Co678 cultures.

Among all combinations of IBA and NAA, genotype Co449 showed the highest rooting frequency (86.67%) with an average roots number per shoot of 16.53 ± 0.02 and root length of 6.90 ± 0.10 cm on half MS medium supplemented with 2.5 mg L⁻¹ IBA. When higher concentration of IBA (5 mg L⁻¹) was used for the same genotype (Co449) on the same media composition, decreased frequency of rooting (66.67%), and fewer number of roots per shoot (7.20 ± 0.10) and shorter root length (3.07 ± 0.31 cm) were recorded. On the other hand, genotype Co678 showed the maximum rooting response (80%) with an average roots number per shoot of 13.17 ± 0.29 and root length of 7.63 ± 0.05 cm on half MS medium supplemented with 5 mg L⁻¹ NAA. When lower concentration of NAA (2.5 mg L⁻¹) was used for the same genotype (Co678) on the same media composition, reduced frequency of rooting (66.67%) and fewer roots per shoot (8.27 ± 0.11) were observed.

The current result is in agreement with other *in vitro*

Tables 6. ANOVA summary of effect of IBA and NAA on *in vitro* rooting and root growth on half MS with sucrose.

Hormone Combination		Co449			Co678		
IBA (mg/L)	NAA (mg/L)	Explants rooted (%)	Number of Roots Per Shoot	Root length (cm) (Mean ± SD)	Explants rooted (%)	Number of Roots per Shoot	Root length (cm) (Mean ± SD)
0	0	0.00 ^m	0.00 ^u ± 0.00	0.00 ^f ± 0.00	0.00 ^m	0.00 ^u ± 0.00	0.00 ^f ± 0.00
0	1.25	33.33 ^{hi}	3.27 ^{p-s} ± 0.06	1.40 ^{o-q} ± 0.10	40.00 ^{gh}	5.27 ^{j-m} ± 0.15	1.80 ^{m-o} ± 0.17
0	2.5	60.00 ^{de}	6.80 ^{hi} ± 0.85	4.13 ^{fg} ± 0.06	66.67 ^{cd}	8.27 ^{fg} ± 0.11	4.67 ^{de} ± 0.15
0	3.75	73.33 ^{bc}	9.83 ^e ± 0.28	5.20 ^c ± 0.36	73.33 ^{bc}	10.9 ^d ± 0.28	5.27 ^c ± 0.35
0	5	80.00 ^{ab}	12.20 ^c ± 0.52	7.10 ^b ± 0.57	80.00 ^{ab}	13.17 ^b ± 0.29	7.63 ^a ± 0.05
1.25	0	46.67 ^{fg}	3.37 ^{o-r} ± 0.06	1.53 ^{n-q} ± 0.12	40.00 ^{gh}	4.30 ^{m-p} ± 0.00	1.90 ^{l-n} ± 0.00
1.25	1.25	73.33 ^{bc}	6.87 ^{hi} ± 0.23	2.07 ^f ± 0.06	66.67 ^{cd}	7.20 ^h ± 0.72	2.57 ^k ± 0.12
1.25	2.5	60.00 ^{de}	9.07 ^{ef} ± 0.12	3.17 ^{ij} ± 0.29	60.00 ^{de}	7.70 ^{gh} ± .26	3.57 ^{h-j} ± .12
1.25	3.75	33.33 ^{hi}	5.30 ^{j-m} ± 0.02	4.40 ^{ef} ± 0.10	40.00 ^{gh}	5.07 ⁱ⁻ⁿ ± 0.67	4.53 ^{ef} ± 0.15
1.25	5	20.00 ^{jk}	3.50 ^{o-r} ± 0.10	2.33 ^{kl} ± 0.15	33.33 ^{hi}	3.50 ^{o-r} ± 0.10	2.13 ^{k-m} ± 0.06
2.5	0	86.67 ^a	16.53 ^a ± 0.02	6.90 ^b ± 0.10	73.33 ^{bc}	11.37 ^{cd} ± 0.55	6.77 ^c ± .25
2.5	1.25	60.00 ^{de}	9.93 ^e ± 0.62	5.30 ^c ± 0.26	60.00 ^{de}	9.63 ^e ± 0.35	5.53 ^l ± 0.21
2.5	2.5	40.00 ^{gh}	6.93 ^{hi} ± 0.81	3.27 ^{h-j} ± 0.25	53.33 ^{ef}	6.00 ^{ij} ± 0.00	3.60 ^{hi} ± 0.10
2.5	3.75	13.33 ^{kl}	4.73 ^{k-n} ± 0.47	3.10 ^{ij} ± 0.10	20.00 ^{jk}	4.40 ^{l-o} ± 0.10	3.27 ^{h-j} ± 0.13
2.5	5	20.00 ^{jk}	3.06 ^{rs} ± 0.12	2.13 ^{k-m} ± 0.06	13.33 ^{kl}	3.07 ^{rs} ± 0.12	2.17 ^{k-m} ± 0.06
3.75	0	73.33 ^{bc}	9.90 ^e ± 0.10	5.07 ^{cd} ± 0.03	60.00 ^{de}	8.30 ^{fg} ± 0.14	4.47 ^{ef} ± 0.06
3.75	1.25	46.67 ^{fg}	5.37 ^{j-l} ± 0.32	4.47 ^{ef} ± 0.15	46.67 ^{fg}	4.37 ^{l-o} ± 0.25	3.76 ^{gh} ± 0.15
3.75	2.5	20.00 ^{jk}	5.53 ^{jk} ± 0.06	3.47 ^{h-j} ± 0.73	40.00 ^{gh}	3.63 ^{o-r} ± 0.15	3.47 ^{h-j} ± 0.06
3.75	3.75	13.33 ^{kl}	3.50 ^{o-r} ± 0.20	2.07 ^{k-m} ± 0.05	33.33 ^{hi}	3.37 ^{o-r} ± 0.06	2.13 ^{k-m} ± 0.06
3.75	5	6.67 ^{lm}	3.17 ^{q-s} ± 0.06	1.93 ^{l-n} ± 0.07	20.00 ^{jk}	3.13 ^{q-s} ± 0.06	1.93 ^{l-n} ± 0.06
5	0	66.67 ^{cd}	7.20 ^h ± 0.10	3.07 ^l ± 0.31	60.00 ^{de}	8.30 ^{fg} ± 0.10	2.53 ^k ± 0.15
5	1.25	33.33 ^{hi}	4.17 ^{n-q} ± 0.57	2.33 ^{kl} ± 0.15	26.67 ^{ij}	3.57 ^{o-r} ± 0.15	2.17 ^{k-m} ± 0.11
5	2.5	20.00 ^{jk}	3.10 ^{rs} ± 0.17	2.10 ^{k-m} ± 0.10	20.00 ^{jk}	3.20 ^{q-s} ± 0.20	2.07 ^{k-m} ± 0.15
5	3.75	20.00 ^{jk}	1.90 ^{pq} ± 0.35	1.30 ^f ± 0.10	13.33 ^{kl}	2.27 st ± 0.15	1.76 ^{m-p} ± 0.12
5	5	6.67 ^{lm}	1.53 ^q ± 0.76	1.10 ^f ± 0.10	6.67 ^{lm}	1.67 ⁱ ± 0.27	1.23 ^q ± 0.02
CV (%)		6.89	5.86	5.28	6.89	5.86	5.28

Means in column with the same letter are not significantly different by Ryan-Einot-Gabriel-Welsh Multiple Range Test at $\alpha=0.05$ significance level.

root induction reports of sugarcane cultures. Bekesha et al. (2002) observed 85% rooting response with an average number of roots per shoot of 15 ± 0.5 and an average root length of 4 ± 0.5 cm on half MS supplemented with 5 mg L^{-1} NAA. Behara and Sahoo (2009) observed 85% *in vitro* rooting on half MS + 2.5 mg L^{-1} NAA with average number of roots per microshoot of 11 ± 1.5 , and average length of roots (cm) 4.0 ± 0.94 . Alam et al. (2003) reported best rooting response at 2.5 mg L^{-1} IBA with 16 numbers of roots per explants having 1.1 cm root length. Mamun et al. (2004) obtained best results of rooting on MS medium supplemented with auxins (NAA + IBA) 0.5 mg L^{-1} for each one. However, the current result is not in accordance with the report of Ali et al. (2008) who reported 100% rooting on medium containing 1.0 mg L^{-1} NAA and 2.0 mg L^{-1} IBA with 2.8 roots per shoot in sugarcane variety CP 77,400 and 3.1 roots per shoot in variety BL-4.

Therefore, half MS medium supplemented with 2.5 mg

L^{-1} IBA + 6% sucrose was the optimum combination for rooting of *in vitro* multiplied shoots of sugarcane genotype Co449 while half MS + 5 mg L^{-1} NAA + 6% sucrose was found to be the optimum medium combination for *in vitro* rooting of shoots of genotype Co678.

Effect of IBA and NAA on rooting of *in vitro* raised sugarcane plantlets on half strength MS with table sugar

ANOVA showed that genotype, IBA, NAA and their interactions had very high significant ($p < 0.0001$) effects on rooting frequency, average root number per shoot and root length (Table 7). Interaction of genotype by IBA by NAA indicated that all the three factors are dependent on each other in influencing *in vitro* rooting of sugarcane cultures in the presence of table sugar. Of the two genotypes, genotype Co449 showed higher rooting frequency

Table 7. ANOVA summary of effect of IBA and NAA on *in vitro* rooting and root growth on half MS with table sugar.

Source of variation	DF	Mean square		
		Rooting rate	Number of roots/shoot	Root length
Gen	1	130.67***	0.82***	0.26***
IBA	4	2644.00***	71.47***	20.56***
NAA	4	3210.68***	23.81***	0.62***
Gen* IBA	4	204.00***	3.77***	0.27***
Gen* NAA	4	197.34***	0.90**	0.32***
IBA* NAA	16	3847.34***	86.24***	22.92***
Gen* IBA *NAA	16	104.00***	1.69***	0.15***
CV (%)		4.04	7.23	5.53

***= Very highly significant ($P \leq 0.0001$) at $\alpha=0.05$ significance level **= highly significant ($P= 0.0009$) at $p<0.05$
 DF= Degree of freedom, Gen = Sugarcane genotypes, IBA=Indole-3-butyricacid and NAA= α -naphthaleneacetic acid.

than genotype Co678 (Table 8) and the variation might be due to their difference in the level of endogenously accumulated PGRs.

In both genotypes, no rooting response was recorded on half MS media devoid of IBA and NAA and rooting occurred in media supplemented with IBA and/or NAA indicating the significance of adding auxin/s in root induction media for rooting of *in vitro* generated sugarcane microshoots (Table 8). Among the different treatment combinations, genotype Co449 showed the highest rooting frequency (86.67%) with an average root number per microshoot of 15.93 ± 0.81 and root length of 7.17 ± 0.15 cm on half MS with 2.5 mg L^{-1} IBA. However, for the same genotype (Co449), an increase in concentration of IBA (5 mg L^{-1}) maintaining the concentration of NAA at 0.0 mg L^{-1} resulted in significantly reduced rooting frequency (60%), lesser number of roots per shoot (7.10 ± 0.10) and shorter root length (3.07 ± 0.12 cm). On the other hand, genotype Co678 showed the maximum root number per microshoot and root length on half MS medium supplemented with 5 mg L^{-1} NAA. On this medium, 80% of the transferred microshoots of genotype Co678 produced the highest root number (13.93 ± 0.81) per microshoot with an average root length of 7.33 ± 0.64 cm. Half MS + 2.5 mg L^{-1} IBA which resulted best rooting in genotype Co449, gave 80% rooting frequency with an average of 12.17 ± 0.76 roots per microshoot and root length of 6.57 ± 0.12 cm in genotype Co678, thus this medium can be taken as the second best medium combination for rooting of this genotype.

The current result agrees with other *in vitro* root induction reports on sugarcane. Khan et al. (2006) stated that the types and concentrations of auxin/s in the rooting media influence the root induction response of sugarcane cultures. Gopitha et al. (2010) observed 80% rooting frequency with mean roots number per microshoot of 9.6 and root length of 3.9 cm on half MS medium supplemented with 5 mg L^{-1} NAA. The same authors reported 54% rooting frequency with an average of 8.8 roots

per microshoot and 3.8 cm root length on half MS medium containing 7 mg L^{-1} NAA indicating higher concentration of auxin(s) result in reduced rooting responses (rooting frequency, root number and root length). Singh (2003) reported the highest rooting frequency (85%) with an average of 13.33 ± 0.6 roots per shoot and root length of 3.8 cm using 5 mg L^{-1} NAA. Many workers also reported that 5 mg L^{-1} NAA was good for rooting of sugarcane microshoots (Shukla et al., 1995; Islam et al., 1996; Gosal et al., 1998; Lal et al., 2001) and more than 5 mg L^{-1} NAA inhibits rooting. Alam et al. (2003) reported best rooting at 2.5 mg L^{-1} IBA with 16 roots per explants having 1.1 cm root length.

On the tops of the current result, it is fair to deduce that half strength MS + 2.5 mg L^{-1} IBA + 6% table sugar is the optimum combination for maximum rooting of *in vitro* generated microshoots of sugarcane genotype Co449 cultures. Whereas, half strength MS + 5 mg L^{-1} NAA + 6% table sugar is found to be the best combination for *in vitro* rooting of Co678 shoots. As can be verified from Tables 6 and 8, for a given genotype and for a given level of IBA and NAA combination, rooting responses on both carbon sources were not significantly different. Demo et al. (2008) also observed an equivalent number of roots per shoot on graded sucrose (7.2) and table sugar (7.5) supplemented media. Thus, it is fair to deduce that table sugar which is locally available and affordable can be used as an alternative carbon source in rooting media also.

Acclimatization of plantlets

For the two genotypes, different acclimatization potential was observed: 80% for genotype Co449 and 86.67% for genotype Co678. Loss of some plantlets might be due to the variation in the method of propagation and environmental factors: Temperature and humidity. The less development of cuticle under *in-vitro* condition and the drop in relative humidity from near 100% in the culture vessels

Table 8. Effect of IBA and NAA on root induction of *in vitro* raised sugarcane plantlets on half strength MS with 6% table sugar.

Hormone combination		Co449			Co678		
IBA (mg/L)	NAA (mg/L)	Explants rooted (%)	Number of Roots Per Shoot	Root length (cm) (Mean ± SD)	Explants rooted (%)	Number of Roots per Shoot	Root length (cm) (Mean ± SD)
0	0	0.00 ^m	0.00 ^u ± 0.00	0.00 ^f ± 0.00	0.00 ^m	0.00 ^u ± 0.00	0.00 ^r ± 0.00
0	1.25	33.33 ⁱ	3.20 ^{o-r} ± 0.10	1.47 ^{opq} ± 0.15	46.67 ^g	5.27 ^{lm} ± 0.15	1.87 ^{no} ± 0.06
0	2.5	53.33 ^f	6.70 ^{ij} ± 0.82	3.73 ^g ± 0.29	6.00 ^e	8.27 ^{efg} ± 0.11	4.77 ^{de} ± 0.06
0	3.75	60.00 ^e	9.43 ^{de} ± 0.51	5.13 ^{cd} ± 0.32	73.33 ^c	10.37 ^d ± 0.21	5.27 ^{cd} ± 0.35
0	5	80.00 ^b	12.33 ^c ± 0.76	7.50 ^a ± 0.26	80.00 ^b	13.93 ^b ± 0.81	7.33 ^a ± 0.64
1.25	0	46.67 ^g	3.40 ^{n-q} ± 0.10	1.33 ^{pq} ± 0.21	40.00 ^h	4.30 ^{m-p} ± 0.00	2.00 ^{mno} ± 0.10
1.25	1.25	73.33 ^c	6.53 ^{ijk} ± 0.50	2.13 ^{lmn} ± 0.15	60.00 ^m	7.20 ^{ghi} ± 0.72	2.67 ^{kl} ± 0.06
1.25	2.5	60.00 ^e	9.50 ^d ± 0.10	3.20 ^{ghi} ± 0.26	6.00 ^e	7.70 ^{ghi} ± 0.26	3.57 ^{gh} ± 0.12
1.25	3.75	33.33 ⁱ	5.23 ^{lm} ± 0.25	4.53 ^{ef} ± 0.15	40.00 ^h	5.10 ^{lm} ± 0.69	4.60 ^{ef} ± 0.26
1.25	5	20.00 ^j	3.50 ^{n-q} ± 0.10	2.47 ^{kml} ± 0.15	33.33 ^j	3.50 ^{n-q} ± 0.10	2.20 ^{lmn} ± 0.10
2.5	0	86.67 ^a	15.93 ^a ± 0.81	7.17 ^a ± 0.15	80.00 ^b	12.17 ^c ± 0.76	6.57 ^b ± 0.12
2.5	1.25	66.67 ^d	9.60 ^d ± 0.79	5.30 ^c ± 0.26	6.00 ^e	9.80 ^d ± 0.62	5.57 ^b ± 0.25
2.5	2.5	40.00 ^h	6.93 ^{hi} ± 0.81	3.33 ^{ghi} ± 0.15	46.67 ^g	6.93 ^{l-o} ± 0.81	3.660 ^{gh} ± 0.10
2.5	3.75	13.33 ^k	4.56 ^{lmn} ± 0.31	3.10 ^{hij} ± 0.10	20.00 ^j	4.40 ^k ± 0.10	3.33 ^{gh} ± 0.12
2.5	5	20.00 ^j	2.93 ^{qrs} ± 0.32	2.20 ^{lmn} ± 0.10	13.33 ^k	3.00 ^{p-s} ± 0.20	2.23 ^{lmn} ± 0.06
3.75	0	73.33 ^c	9.27 ^{def} ± 0.23	5.13 ^{cd} ± 0.06	66.67 ^d	9.33 ^{def} ± 0.12	4.47 ^{ef} ± 0.06
3.75	1.25	46.67 ^g	5.23 ^l ± 0.32	4.47 ^{ef} ± 0.15	46.67 ^g	5.37 ^{klm} ± 0.25	4.23 ^f ± 0.12
3.75	2.5	20.00 ^j	5.37 ^{klm} ± 0.32	3.47 ^{gh} ± 0.06	40.00 ^h	5.63 ^{ikl} ± 0.15	3.47 ^{gh} ± 0.06
3.75	3.75	13.33 ^k	3.50 ^{n-q} ± 0.20	2.10 ^{mno} ± 0.00	3.33 ⁱ	3.50 ^{n-q} ± 0.20	2.20 ^{lmn} ± 0.10
3.75	5	6.67 ^l	3.07 ^{p-s} ± 0.06	1.93 ^{mno} ± 0.06	13.33 ^k	3.13 ^{o-s} ± 0.06	1.93 ^{mno} ± 0.06
5	0	60.00 ^e	7.10 ^{ghi} ± 0.10	3.07 ^{hij} ± 0.12	66.67 ^d	8.16 ^{fgh} ± 0.06	2.90 ^{ijk} ± 0.26
5	1.25	33.33 ⁱ	4.16 ^{n-q} ± 0.56	2.17 ^{lmn} ± 0.16	20.00 ^j	3.50 ^{n-q} ± 0.10	2.20 ^{lmn} ± 0.10
5	2.5	20.00 ^j	3.17 ^{o-s} ± 0.15	2.07 ^{mno} ± 0.06	13.33 ^k	3.17 ^{o-s} ± 0.15	2.10 ^{mno} ± 0.10
5	3.75	20.00 ^j	1.90 st ± 0.35	1.80 ^{nop} ± 0.10	13.33 ^k	1.90 st ± 0.35	1.93 ^{mno} ± 0.06
5	5	6.67 ^l	1.40 ^t ± 0.53	1.27 ^q ± 0.21	6.67 ^l	1.03 ^{rs} ± 0.06	1.17 ^q ± 0.12
CV (%)		4.04	7.23	5.53	4.04	7.23	5.53

Means in column with the same letter are not significantly different by Ryan-Einot-Gabriel-Welsh Multiple Range Test at $\alpha=0.05$ significance level.

to much lower values in the poly house might result in excessive water loss and death (Biradar et al., 2009). The current result is in agreement with the report of Ali et al. (2008) who declared 70-80% greenhouse acclimatization potential of *in vitro* generated sugarcane cultures. Biradar et al. (2009) also reported 72% survival rate of micropropagated plantlets.

Conflict of Interests

The author(s) have not declared any conflict of interests.

Conclusion

Lack of a steady supply of good planting material is one of the bottle necks for the exploration of the potential of sugarcane in Ethiopia. Mass propagation of sugarcane through shoot tip culture ensures quick availability of genetically uniform (true to type) diseases free planting

materials within short period of time. In the present study, an effective protocol for subsequent *in vitro* plantlets multiplication from shoot tip explants was developed for sugarcane genotypes Co449 and Co678. Accordingly, irrespective of the type of carbon source (3% sucrose or 4% table sugar) used in the MS medium, the combination of 2 mg L⁻¹ BAP + 0.25 mg L⁻¹ Kin is found to be the best combination for shoot multiplication of genotype Co449 while 2 mg L⁻¹ BAP + 0.5 mg L⁻¹ Kin is the optimum combination for shoot multiplication of genotype Co678. Regarding *in vitro* rooting, half MS medium + 2.5 mg L⁻¹ IBA is found to be the best combination for rooting of microshoots of genotype Co449 while half MS + 5 mg L⁻¹ NAA is the best media combination for maximum rooting of microshoots of genotypes Co678. The study also revealed that shoot multiplication and rooting responses on sucrose or table sugar supplemented media are not significantly different. Hence, it is fair to deduce that we have developed a cost effective protocol, which can use table sugar instead of costly graded sucrose for *in vitro*

mass propagation of two commercial sugarcane genotypes grown in Ethiopia: Co449 and Co678. Hence, to make developing countries like Ethiopia beneficiary of micropropagation technology, the utilization of such locally available and economically reasonable resources in place of the expensive ones is best alternative.

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Full Length Research Paper

Fertigation of stillage in the culture of brown and golden linseed (*Linum usitatissimum*)

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In Brazil, the stillage is mainly used in fertigation of sugarcane plantations, however, little is known about its effect on the nutritional supplementation and irrigation cultivation of linseed. Because of the shortage of work in the area, the present study aimed to evaluate the development of the culture of brown and golden linseed submitted to fertigation of stillage. The experiment was conducted in the field, at the Federal University of Paraná in Palotina/PR in the agricultural year of 2013, on a eutrophic Red Latosol. The adopted lineation was completely randomized in split plots, where the plots were composed by the two varieties (brown and gold), and the sub-plots by the ratios (100% stillage; 50% stillage and 50% water; 100% control water). Fertigation of stillage in the culture of brown and golden linseed presents positive results on plant height, dry and fresh weight, capsules number and productivity; except for the number of branches (100% is higher). It was observed that between the two varieties, the golden linseed showed better development with the applied treatment.

Key words: Fertigation, energy crops, agro industrial residues.

INTRODUCTION

Linseed (*Linum usitatissimum*) is the flax seed, commonly used in culinary and ingested with its husk. The earliest reports of the use of this oilseed are dated of the time of Mesopotamia. Its origin is Asian, though its consumption is worldwide, with common use mainly in Europe and North America (Bombo, 2006). Linseed is

one of the most important cultivated plants worldwide in terms of vegetation cover and oil content. Its pie, byproduct of the oil extraction process, is very nutritious and can be used in animal feed. Furthermore, the linseed has high presence of lignans, especially secoisolariciresinol diglucoside (SDG), which are related to reduced risk of

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cardiovascular diseases and are capable of inhibiting the development of diabetes, and are also important in the development of the central nervous system and important in the treatment of certain cancers (FAO, 2008; Oomah and Mazza, 1993; Westcott and Muir, 2003; Harbige et al., 2008).

The oilseed, among infinitude of functional foods, stands out for presenting one of the most interesting sources of essential fatty acids ω -3 and ω -6, besides several phenolic compounds, nutrients and fiber that act as antioxidants in the body (Mayes, 1994; Hall et al., 2006). There are two main varieties of linseed, the brown and the golden linseeds. Their seeds do not differ in their chemical composition in large proportions, since both have high levels of lignans and dietary fiber featuring more than 50% of phenolic in their composition. The golden linseed is better adapted to cold climates, having higher levels of protein in their composition when compared to brown linseed (Lima, 2008; Marques, 2008). In Brazil, the cultivation of linseed remains mainly in Rio Grande do Sul, being maintained by descendants of Polish and German immigrants. Linseed requires climates with relatively low temperatures for flowering, around 0 to -2°C . The plant does not require intensive cultivation and can be grown in crop rotation system on degraded lands in order to retrieve the soil depletion to erosion. Its planting usually occurs in the months of May and June (Soares et al., 2009; Vieira et al., 2012). The current national agriculture needs to adapt itself to new circumstances and complexities in an environment of extreme competitiveness, so that producers need to improve their techniques with better possible utilization of productive resources. Irrigation techniques are directly linked to agricultural production (Oliveira et al., 2012).

Fertigation is considered as fertilization technique using the water itself in order to bring nutrients to the soil. It can occur through the use of diluted commercial fertilizers or through the use of organic waste, and in large part; the technique is used in order to fertilize the soil efficiently and inexpensively, reducing the problem of proper treatment and disposal of nutrients (Dalri and Cruz, 2002). The stillage is a byproduct of the ethanol industry with dark brown color and high organic matter content (50 to 150 g L^{-1}), usually presenting relatively low pH values (3.5 to 5) (España-Gamboa et al., 2011; Waliszewski et al., 1997). Its generation shows the mean proportion of 13 L for each liter of ethanol produced (Carvalho and Silva, 2010).

According to data from the National Supply Company (Conab), in 2012, sugarcane production reached 490 million tons, and its expected growth of 7.2% for the coming years. Due to high production of waste in the production process of ethanol, several alternative designations of this material that are not harmful to the environment are studied. The first studies on fertigation of stillage in Brazil started in the 50s, becoming common in

cane refineries. The process of fertigation with stillage is the infiltration of residue in the soil by irrigation channels, normally destined to sugarcane (Camargo et al., 2009). Its application decreases costs with chemical fertilizers and it is an alternative to the use of natural resources, avoiding the downloading of this material in rivers, while it is used on agricultural land (Laime et al., 2011; Gianchini and Ferraz, 2009). Diverging of studies that point the direct application of stillage related to salinization, leaching of metals presents in the soil to groundwater and changes in soil quality, studies developed by Penatti et al. (1999) show that volumes up to $300\text{ m}^3/\text{ha}$ of stillage with the presence up to 4 kg of potassium per m^3 , do not alter the physicochemical and biological soil properties, regardless of their composition. When deposited on the soil, the stillage also ensures fertility due to the increased availability of some ions, increased cationic exchange capacity, increased water retention capacity and improvements in the physical structure of the soil. Due to its rich composition in nutrients, especially potassium, most Brazilian distilleries use this residue in fertigation of sugar cane plantations (Silva et al., 2007). From this information and due to the lack of studies focused on the study of fertigation of linseed, the present study aimed to evaluate the development of the culture of brown and golden linseed with stillage application in different proportions from the analysis of various parameters of plants.

MATERIALS AND METHODS

The experiment was conducted in the field during the period from May to September in the agricultural year of 2013, in the area of the Federal University of Paraná in Palotina/PR, located in the following coordinates: latitude $24^{\circ}18'$ S, longitude $53^{\circ}55'$ W and elevation of 310 m. The city has a eutrophic Red Latosol soil, with subtropical climate (Cfa), according to the Köppen classification, with no definite dry season. Its average annual temperatures vary between 17 and 19°C with hot summers and average rainfall well distributed throughout the year between 1,200 and 2,000 mm per year (IAPAR, 2006). The characterization of the soil where the experiment was carried out can be found in Table 1. The adopted lineation was completely randomized in split plots, where the plots were composed by the two varieties (brown and gold), and the sub-plots by the ratios (100% stillage; 50% stillage and 50% water; 100% control water). The plot was consisted by 9 rows of 5 m of length. The applications were performed every 4 days in volume of 8 L/m^2 . The stillage used in the fertigation was from pilot plant of ethanol production, and must be fermented had only yeast *Saccharomyces cerevisiae* added, without addition of any additive of optimization process. The characterization of the stillage in nutrients can be found in Table 2.

According to Soares et al. (2009), the culture of linseed occurs in the fall, in the months of May and June and its harvesting in November, December and January. From these information, the linseed was planted at the beginning of May and harvested at the beginning of November, and the plants were harvested in the morning and after weighing the fresh weight of aerial portion and fresh weight of the root, the biomass was placed in a greenhouse with forced air circulation, with the temperature of $65^{\circ}\text{C} \pm 2$ for determination of dry weight of aerial portion and dry weight of the

Table 1. Soil composition where the experiment was conducted.

Ca	Mg	K	Al (Cmolc/dm ³)	H+Al (Cmolc/dm ³)	Sum of bases	CCE	C	Microorganism (g/dm ³)
5.67	1.84	0.72	0.00	4.28	8.23	12.51	20.74	35.67
Base Saturation (%)			P	Fe	Mn	Cu	Zn	pH CaCl ₂
65.8			12.96	18.48	146.38	8.86	5.01	5.10

CCE: Capacity of cations exchange.

Table 2. Characterization of the sugar kane stillage used in the linseed fertigation.

Nutrient	N	P	K	Ca	Mg	pH
	g/L					
	14.35	0.02	0.85	-	-	5.76

root. After this procedure, the samples were reweighed in a balance of semi-analytical accuracy. The greenhouse with forced air circulation step was repeated until constant weight was reached. Plant height, number of branches, number of capsules and productivity kg ha⁻¹ were recorded. Statistical analysis (ANOVA) was performed using the statistical software ASSISTAT 7.6 beta and the comparison between treatment means was performed by applying the Tukey test at 1 and 5% probability and regression analysis at 1 and 5% probability.

RESULTS AND DISCUSSION

According to Table 3, the effect of varying the concentration of stillage in the analyzed variables can be verified. Fertigation with stillage influenced the components of linseed crop production. It could also be shown in Table 3 that the interaction between varieties and stillage concentration does not significantly interfere in fresh weight of the plant, and in the number of branches and capsules. From the unfolding of variables that showed interaction between the factors (Table 4), it can be observed that a significant difference between the grain productions of the two varieties of linseed with a higher productivity for the golden linseed where stillage was applied in proportions of 100%. Nevertheless, it is important to note that the brown linseed production increased by around 43% from the treatment without stillage to the treatment with 50% in waste composition, it could be a noted superior production of the golden linseed in these concentrations. From the unfolding of variables that showed interaction between factors (Table 4), it can be observed that plant height was positively influenced by the application of stillage. The concentrations (50 and 100%) did not differ between each other, regardless of variety. It can be observed that the fertigation with stillage positively influenced the dry matter production. The highest concentra-

tions of stillage (50 and 100%) did not provide dry matter accumulation by the plant, regardless of the variety, the same was observed by irrigation only with water. The number of branches per plant (Table 4) followed the same pattern observed for plant dry matter, where the highest concentrations and only irrigation did not differ between cultivars. Productivity of the golden linseed (648.42 kg ha⁻¹) was superior to the brown cultivar (407.10 kg ha⁻¹) for the 100% fertigation concentration. When the linseed was subjected only to irrigation (0% stillage concentration), and it can be verified by superior results for the golden variety. De Paula et al. (1992) evaluated the production of onion crop with treatments with stillage, it was noted that the use of fertigation residue assured significant increase in vegetable production per unit area. The application of stillage from 200 to 400 m³/ha provides superior production of the pineapple culture, and the stillage replaces KCl as K source (De Paula et al., 1999). In works that evaluated the initial development of oilseed plants like groundnut, sunflower and castor bean, it was found that for the first two crops, the application of stillage was detrimental to emergence and seedling development, whereas for castor bean, the interference of stillage was positive in the variables that are related to seedling vigor (Ramos et al., 2008). Works of fertigation with stillage are commonly found for the sugar cane culture. In a study evaluating the production of sugar cane with fertigation of stillage, it was concluded that, concentrations at doses of 300 and 450 m³ ha⁻¹, the production of culms was increased, furthermore, doses of 300 m³ ha⁻¹ provided better rooting in layer of 0.25 and 0.50 m deep (Medina et al., 2002). A work performed by Barbosa et al. (2012) using fertigation of stillage for sugar cane culture complementing the need for potassium; irrigation by surface drip also favored the production of culms. The same results are obtained in a study by Oliveira et al. (2009).

Barbosa et al. (2012) further states with experimentation that the use of fertigation of stillage on the sugar cane culture promotes alterations in the number of tillers and leaf area index, compared to non-irrigated cultivation, regardless of fertigation management. In the culture of cane, the stillage promotes reduction of sugar concentration; however it provides vigorous vegetative growth and

Table 3. Effect of the variation of stillage concentration on analyzed variables of golden and brown linseed.

Factor varieties	Height (cm)	Fresh weight (g)	Dry weight (cm)	Branch number	Capsule number	Productivity (Kg/ha)
Golden (A)	73.50 ^b	14.45 ^a	4.07 ^a	3.96 ^a	27.71 ^b	383.64 ^b
Brown (B)	79.42 ^a	15.03 ^a	3.91 ^a	3.58 ^a	36.25 ^a	503.05 ^a
Stillage (%)						
0	62.69 ^b	7.92 ^b	2.62 ^b	3.19 ^b	17.06 ^c	363.88 ^c
50	83.75 ^a	17.01 ^a	4.53 ^a	3.94 ^{ab}	31.19 ^b	438.39 ^b
100	82.94 ^a	19.28 ^a	4.77 ^a	4.19 ^a	47.688 ^a	527.76 ^a
CV %-A	8.47	25.40	21.95	21.02	26.11	7.98
CV %-B	8.70	31.62	23.78	26.91	33.61	5.95
Varieties	10.0162 ^{**}	0.2857ns	0.2404ns	2.6872 ns	12.5571 ^{**}	51.3021 ^{**}
Vinasse	51.4310 ^{**}	26.6035 ^{**}	24.7753 ^{**}	4.2081 [*]	32.5462 ^{**}	58.0631 ^{**}
Int. AxB	4.3320 [*]	1.1587ns	0.0129 [*]	3.1561ns	3.3211ns	58.8071 ^{**}

Averages in each column followed by the same letter do not differ significantly between each other by the Tukey test, at 5% probability. VC (%) = Variation coefficient. (**) = Significant at 1% probability. (*) = significant at 5% probability. (ns) = not significant.

Table 4. Unfolding of interaction for height, dry weight and productivity.

Linseed	0	50% Stillage	100% Stillage
Height (cm)			
Brown	55.75bB	83.13aA	81.63aA
Golden	69.63aB	84.38aA	84.25aA
Dry weight (g)			
Brown	2.67aB	4.62aA	4.81aA
Golden	2.57aB	4.44aA	4.73aA
Productivity (Kg/ha)			
Brown	271.14bC	472.67aA	407.10bB
Golden	456.61aB	404.12bB	648.42aA

Using small letters for columns and big letters for rows, averages followed by the same letter do not differ significantly between each other by the Tukey test, at 5% probability.

dry mass gain (Korndorfer, 1990). The stillage presents in its composition high concentrations of potassium. In a work by Rossetto et al. (2012), analyzing the influence of applying different levels of potassium in cultures of golden and brown linseed, it was proven that linseed shows significant response to treatments with macro-nutrient, and that the golden linseed obtained better performance. Corroborating with the data obtained in the study with the treatments of stillage, Turner (1987) states that potassium is an element that elevates the fiber content of linseed. Potassium is present in plants in concentrations near the nitrogen, and for a good development of the plant, potassium contents are between 2 and 5% of the dry weight, depending on each species. Potassium fertilization as well as phosphorus directly helps to increase the production of roots, flowers and leaves, besides affecting the production of active principles (Meurer, 1995; Bevilacqua et al., 2007). Lewis et al. (1991) also states

that with certain levels of potassium, leaf area of different oilseed cultures is increased, as well as stem thickness, number of grains per spike and oil content. According to Khajani et al. (2012), combined applications of nitrogen, phosphorus and potassium bring positive impacts on production components of grain and on the linseed oil. Some cultivars of linseed, when treated with potassium fertilization, show resistance to diseases like fusarium.

Conclusion

With the present study, it can be concluded that fertigation of stillage in the culture of brown and golden linseed have significant positive results in all analyzed variables. It is important to point that between the two varieties, the golden linseed performed better in grain production than the brown linseed variety.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Effect of high doses of equine chorionic gonadotrophin (eCG) treatments on follicular developments, ovulation and pregnancy rate in boer goats

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The aim of this study was to determine the effects of the superovulatory technique using equine chorionic gonadotrophin (eCG) on follicle response, ovulation and pregnancy rate in Boer goats. Twenty nine (29) does were divided into three groups, G1 (n = 11), G2 (n = 8) and G3 (control, n = 10). All groups had their estrus synchronized by the use of controlled internal drug release (CIDR) containing 0.3 g progesterone for 18 days. Twenty-four hours prior to CIDR removal, all animals were intramuscularly injected with different eCG doses: does in G1, G2 and G3 received 600, 800 and 1000 IU eCG, respectively. Follicular activity was determined once a day for four consecutive days by ultrasonographic monitoring starting at eCG treatment (day 17) in all groups. The number of corpora lutea were assessed on day seven after estrus to calculate ovulation rate, whereas the pregnancy diagnosis was detected on 30 days post mating. Follicles response resulted in significant differences ($P < 0.05$) only under small size follicles but not significant difference ($P > 0.05$) on number of follicles under medium and large size follicles among treatments. Ovulation rate recorded a significant difference ($P < 0.05$) among treatments after seven days post estrus with the highest rate at 2.3 ± 0.3 , 1.6 ± 0.2 and 1.4 ± 0.1 for G2, G3 and G1, respectively. Meanwhile, pregnancy rate that showed the highest recorded was 50, 45.5 and 12.5% for G3, G1 and G2, respectively. The results concluded that there was no significant difference on follicle number recorded among treatments except for small size follicle numbers on days 19 and 20. Meanwhile, we concluded that 800 IU eCG was the best treatment resulting in the highest ovulation rate. Different doses of eCG however did not influence the pregnancy rate in superovulated does.

Key words: Equine chorionic gonadotrophin, follicular, ovulation, pregnancy, estrus synchronization, goat.

INTRODUCTION

The optimization of reproductive performance is one of the main facts that assure high productivity on goat farms. This requires that the management practices take into account the physiology and behavior of the animals since environmental, managerial and sanitary aspects interfere with fertility and can impair it. Indeed, reproduction could be considered a "luxury" function and the female appears

able to feel whether the conditions are too severe and risky for a successful reproductive cycle (Fringgens, 2003). Multiple ovulation and embryo transfer (MOET) is widely used to increase genetically superior offspring produced from selected females (Greyling, 2002). Ovarian superstimulation in domestic animals may thus be used to increase the number of developmentally competent

oocytes for *in vivo* or *in vitro* embryo production (Malhi et al., 2008). This variation may be due to both extrinsic (equine chorionic gonadotrophin (eCG) treatment and follicle stimulating hormone (FSH) preparation, mode of administration or the dosage regimens) and/or intrinsic (ovarian status, genetic variation) factors (Cognie et al., 2003; Gonzales-Bulnes et al., 2004; Shipley et al., 2007).

Superovulatory procedures using eCG commonly used high dose of eCG after synchronizing their estrus cycles with controlled internal drug release (CIDR). This procedure agreed with a previous research (Holtz, 2005) which they reported that, in goats, superovulatory treatment typically consists of a combination of estrous cycle control (usually involving application of progestagen implants), with an elevated dose of gonadotropin, to induce the ovary to release more than the typical number of oocytes. The eCG traditionally termed "pregnant mare serum gonadotropin" (PMSG) is an exogenous gonadotropin which can stimulate follicular growth and subsequently the number of ovulation (ewe on day 12 of the cycle) (Cumming, 1975). PMSG resembles pituitary FSH and luteinizing hormone (LH) in that it is a glycoprotein. It can be said to be the complete gonadotropin, since it is able to induce follicle growth, estrogen production, ovulation, luteinization and progesterone synthesis (Cahill, 1982). PMSG is administered as a single subcutaneous or intramuscular injection given one day prior to the last synchronization treatment. In goat, 1000 IU PMSG is given on day 17 of the oestrous cycle (Bondurant, 1986; Drost, 1986) or 2 days before progesterone sponge removal or cessation of daily (12 mg/day) progesterone injection. Several reports claim good results using doses of eCG that ranged from 200 to 600 IU Greyling and Van Niekerk, 1990; Ritar et al., 1994; Menegatos et al., 1995; Freitas et al., 1996; Selvaraju and Kathiresan, 1997).

The present study aimed to compare the lower dosages of eCG at 600 and 800 IU, compared to 1000 IU which eventually improve reproductive efficiency as well as reducing cost of superovulatory treatment in the goats.

MATERIALS AND METHODS

The experiment was conducted in a total of 29 healthy pluriparous Boer does, aged from three to five years and body weight between 45 and 65 kg maintained indoors at the experimental farm of Kampung Kuala Pah Breeding Goat Center, owned by Department of Veterinary and Services (DVS), Malaysia. The animals were randomly divided into three groups. Group 1 consisted of 11 does, group 2 with eight does and group 3 (control) with 10 does. Animals were housed in an animal shed (6.1 × 6.1 m) built approximately 3 m

above the ground level. The goat house was located at the hill top surrounded by open paddock. All does were kept indoors and fed twice daily. In the morning, animals were fed with *Bracharia humidicola* (Rendle) and *Panicum maximum* (Guinea). In the evening, commercial concentrates (Biopalma[®]) (Crude protein ≤ 14.9%, crude fiber ≤ 26.1%, crude fat ≤ 5.1%, calcium ≤ 0.72%, phosphorus ≤ 0.36 % and metabolisable energy ≤ 9.06 MJ/kg) were given to the animals at 450 g/doe/day. Mineral blocks and water were provided *ad libitum* to the animals.

Does in the three experimental groups were followed superovulation protocol by synchronizing with controlled internal drug release (CIDR[®]) device which contained 0.3 g of progesterone and left intravaginally for 18 days. The day of CIDR insertion was considered as day 0. Twenty four hours prior to CIDR removal, superovulatory treatments were given using eCG. Does in groups 1, 2 and 3 received a single intramuscular injection of 600, 800 and 1000 IU eCG (Foligon[®], Intervet, The Netherlands), respectively.

Ovarian images were obtained with a B-mode ultrasound scanner (ALOKA SSD-500[®], Tokyo, Japan) equipped with a 5 MHz linear array transducer. During scanning, goats were restrained in a wooden chute in a standing position. Before scanning, feces were removed as much as possible by hand and some carboxymethylcellulose gel placed on the transrectal probe. Then, the lubricated transrectal probe was inserted into the rectum. When the urinary bladder was surpassed and the uterine horns were located, the probe was rotated laterally clockwise for 90° and counter-clockwise for 180° to evaluate both ovaries and their structures as described (Ginther and Kot, 1994). The ovaries were scanned in several planes to identify all visible follicles that are >1 mm in diameter. Follicles that were more than 3 mm were counted and grouped into one of the following classes: small (3 to < 4 mm), medium (4 to < 5 mm) and large (≥ 5 mm) follicles, following the previous study (Menchaca and Rubianes, 2002). Follicular development was observed once a day for four consecutive days starting on the day of eCG treatment. Twenty-four hours after CIDR removal, a buck was mixed with the females in each group. The information pertaining to the mating time such was recorded.

The ovarian response in terms of number corpora lutea (CLs) was assessed by 5 MHz linear probe attached to an ultrasound scanner (ALOKA SSD-500, Tokyo, Japan) seven days after the onset of estrus. We recorded the number of CL's with the antral follicles ≥ 3 mm in diameter. Meanwhile, pregnancy rate was determined by pregnancy diagnosis on 30 days post mating.

Statistical analysis was performed using Predictive Analytics SoftWare (PASW[®]) version 17.0. All follicles were grouped as described earlier. The effects of treatment on number of follicles and ovulation rate were analyzed by analysis of variance (one way ANOVA). Meanwhile, percentage calculation and Chi-square analysis were conducted to measure the effect of treatments on pregnancy rate. Data were expressed as mean ± standard error of mean (S.E.M) and differences were considered to be statistically significant at $P < 0.05$.

RESULTS

The follicle response of Boer does following estrus synchronization is shown in Table 1. The patterns of follicular

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Abbreviations: CIDR, Controlled internal drug release; CL, corpus luteum; DVS, department of veterinary and services; eCG, equine chorionic gonadotrophin; FSH, follicle stimulating hormone; g, gram; LH, luteinizing hormone; mg, miligram; MHz, Megahertz; MOET, multiple ovulation and embryo transfer; PASW[®], predictive analytics software; PMSG, pregnant mare serum gonadotropin.

Table 1. Follicular development of Boer does following estrus synchronization (Mean \pm S.E.M).

Day	Size	G1 (n = 11)		G2 (n = 8)		G3 (n = 10)	
		LO	RO	LO	RO	LO	RO
Day 17	S	1.3 \pm 0.3	4.0 \pm 0.5	2.1 \pm 0.5	2.0 \pm 0.0	2.3 \pm 0.2	2.0 \pm 0.5
	M	1.8 \pm 0.3	1.2 \pm 0.2	1.5 \pm 0.2	1.0 \pm 0.0	1.5 \pm 0.2	1.4 \pm 0.2
	L	1.0 \pm 0.0	1.2 \pm 0.2	5.0 \pm 0.0	1.5 \pm 0.5	1.4 \pm 0.4	2.3 \pm 0.3
Day 18	S	2.0 \pm 0.7	1.7 \pm 0.4	1.0 \pm 0.0	2.0 \pm 0.0	2.6 \pm 0.3	3.0 \pm 2.0
	M	1.5 \pm 0.2	1.6 \pm 0.3	1.2 \pm 0.2	1.0 \pm 0.0	3.4 \pm 1.2	4.3 \pm 1.0
	L	2.2 \pm 0.4	1.5 \pm 0.5	1.7 \pm 0.7	2.0 \pm 0.3	3.0 \pm 0.7	2.2 \pm 0.9
Day 19	S	1.2 \pm 0.1 ^a	3.1 \pm 0.3	1.7 \pm 0.2 ^{a,b}	1.8 \pm 0.3	2.5 \pm 0.6 ^b	3.5 \pm 1.5
	M	1.8 \pm 0.3	1.6 \pm 0.2	2.0 \pm 0.6	1.0 \pm 0.0	2.4 \pm 0.6	2.0 \pm 0.4
	L	1.4 \pm 0.2	2.0 \pm 0.3	2.1 \pm 0.4	1.6 \pm 0.6	3.0 \pm 0.7	1.8 \pm 0.4
Day 20	S	1.4 \pm 0.2	1.0 \pm 0.0 ^x	2.0 \pm 1.5	1.0 ^x \pm 0.0	1.7 \pm 0.2	2.5 \pm 0.2 ^y
	M	1.2 \pm 0.1	1.1 \pm 0.1	2.0 \pm 0.6	1.5 \pm 0.5	2.1 \pm 0.5	1.4 \pm 0.2
	L	2.0 \pm 0.0	1.5 \pm 0.3	1.6 \pm 0.4	2.1 \pm 0.4	1.2 \pm 0.2	2.4 \pm 0.5

^{a, b}Values with different superscript in the same row differ significantly at $P < 0.05$. ^{x, y}Values with different superscript in the same row differ significantly at $P < 0.05$. S, Small size follicle; M, medium size follicle; L, large size follicle; LO, left ovary; RO, right ovary.

growth within different size categories indicate the duration of exogenous gonadotrophin stimulus. The shift from the numerical superiority of the smaller follicle category to the superiority of the next higher one at the attainment of the next phase of the estrus period (pre-estrus to estrus, or estrus to post estrus) represents the end of recruitment, and therefore the end of the exogenous gonadotrophin action. Regarding the experiment, after an hour super-ovulatory treatment given, the highest mean number of follicles found was 4.0 ± 0.5 , under small size category in right side ovary of G1. However, mean number of follicles for small, medium and large size category at left and right side ovary was not significant ($P > 0.05$) among group treatments on day 17.

Twenty four hours after superovulatory treatment, the highest mean number of follicles recorded was from medium size categories at 4.3 ± 1.0 from G3 on right side ovary, followed by G1 (2.2 ± 0.4) and G2 (2.0 ± 0.3), on left and right ovary, respectively. Only G1 resulted in an increasing number of follicle on large size categories in both side ovary compared to others group. On day 18, no significant difference ($P > 0.05$) was found on number of follicles between group treatments at left and right side ovary on all size categories.

Twenty four hours after CIDR removal, the highest mean number of follicles recorded on day 19 was in G3 (3.5 ± 1.5) followed by G1 (3.1 ± 0.3) both under small size category and G2 (2.1 ± 0.4) under large size category. The mean number of follicles are significantly ($P < 0.05$) higher among group treatments in left ovary on small size categories at 2.5 ± 0.6 , 1.7 ± 0.2 and 1.2 ± 0.1 for G3, G2 and G1, respectively. However, no significant difference ($P > 0.05$) on number of follicles was recorded

in right ovary under small size categories at the same time. We also recorded no significant difference ($P > 0.05$) under medium and large size categories on both sides of the ovary on day 19.

On day 20, 48 h after CIDR removal, a highly significant difference ($P < 0.05$) in number of follicles was recorded with G3 at 2.5 ± 0.2 follicles, followed by G2 (2.0 ± 1.5) and G1 (1.4 ± 0.2) under small size categories. The highest number of follicles recorded from G1 and G2 on day 20 was 1.5 ± 0.3 and 2.1 ± 0.4 , respectively both under large size follicles. Table 2 shows the ovulation rate of does administered with CIDR + eCG. In this study, 11 (100%) out of 11 does in G1, eight (100%) out of eight does in G2 and 10 (100%) out of 10 does in G3 ovulated during estrus. The highest mean numbers of CL at the left side ovary was from G2, which is 1.3 ± 0.1 . However, the mean numbers of CL on the left ovary was not significantly different ($P > 0.05$) compared with G1 (1.11 ± 0.11) and G3 (1.00). Meanwhile, the highest mean numbers of CL found on the right ovary was from G2 at 1.3 ± 0.2 , followed by G3 and G1 at 1.3 ± 0.1 and 1.0, respectively. This result shows that the ovulation rate from right ovary was not significantly different ($P > 0.05$) among group treatments. The highest numbers of CL recorded in the right side ovary was two from G2 and G3; meanwhile the lowest CL recorded was 1 from G1.

Table 3 shows the result of does that conceived by natural mating. Out of 21 estrus does, 11 (52.4%) does were pregnant. The highest pregnancy rate in this study was from G3 at 71.4% followed by G1 (55.5%) and G2 (20%). Only G2 and G1 resulted more than 50% pregnancy rate. In contrast, G1 and G2 recorded the highest non pregnant rate, with four does in each group,

Table 2. Ovulation rate after following 18 days CIDR treatment with eCG injection on day 17.

Parameter	G1	G2	G3	Total
Number of females synchronized	11	8	10	29
Number of does ovulated	11 (100%)	8 (100%)	10 (100%)	29 (100%)
Mean number of CL on left ovary (range)	1.1 ± 0.1 (1-2)	1.3 ± 0.1 (1-2)	1.0 ± 0.0	
Mean number of CL on right ovary (range)	1.0 ± 0.0	1.3 ± 0.2 (1-2)	1.3 ± 0.1 (1-2)	
Mean number of CL per doe (range)	1.4 ± 0.1 ^a (1-2)	2.3 ± 0.3 ^b (1-4)	1.6 ± 0.2 ^{a,b} (1-3)	

^{a, b}Values with different superscript in the same row differ significantly at P<0.05. Mean ± S.E.M.

Table 3. Pregnancy rate following 18 days CIDR treatment with eCG injection on day 17.

Parameter	G1 (n=11)	G2 (n=8)	G3 (n=10)	Total
Number of does in estrus	9	5	7	21
Number of does that did not display oestrus	2	3	3	8
Number of does pregnant	5 (55.5%)	1 (20%)	5 (71.4%)	11 (52.4%)
Number of does aborted	0	0	2	2
Gestation period (days)	144.4 ± 7.5	145.0	145.3 ± 6.5	

Mean ± S.E.M.

after estrus. Only two does from G3 failed to become pregnant in this study. However, we observed two pregnant does from G3 has aborted on midway stage pregnancy. According to the mean gestation period recorded, G3 showed the longest period with 145.3 ± 6.5 days. Meanwhile, G2 and G1 recorded 145 and 144.4 ± 7.5 days gestation period, respectively. Thus, the proportion of gestation period was not influenced by eCG doses.

DISCUSSION

On the first day of eCG treatment, the highest mean number of follicles was from G1 at 4.0 ± 0.5 in small size category. This finding was in line with previous study (Francicco Carlos de Sousa et al., 2011) which found 7.1 ± 0.9 follicles in 3 to 4 mm size at the ovarian stimulation day. Another study (Riesenberg et al., 2001) also concurred to our study which reported 8 h after eCG treatment given, mean number of follicles that range 0.3 and 0.4 cm was 2.7. The highest mean number of follicles found on day two after eCG treatments in the current study was 4.3 ± 1.0 from G3 under medium size category. Our finding is higher than previous study (Kermani et al., 2012) which recorded 1.6 ± 0.4 follicles (≥ 4 mm) at the same time after receiving 850 IU eCG. However, the same findings are more similar with the present study after comparing the highest mean number of follicles (4 ≤ 5 mm) found in G2 (800 IU) at 1.2 ± 0.2 at the same period.

The present study shows the highest mean number of 5 follicles found on day three after eCG treatment was 3.5 ± 1.5 from G3 in small size category. This is comparable

with previous findings by Kermani et al. (2012) which recorded 3.5 ± 0.6 mean number of follicles at the same size category. Four days after eCG treatments, our study recorded the highest mean number of follicle at 2.5 ± 0.2 from small size category from G3. This finding agrees with previous study by Ali (2007) who found the highest follicles on day four after eCG treatment was 2.5 follicles. These findings are also close to the previous study by Salehi et al. (2010) who showed that the administration of eCG and FSH caused growth and development of the small follicles present in the ovaries. Subsequently, the large follicles during estrus will ovulate and transform to mature CL's.

The ovulation rate in our study showed that all (100%) does ovulated with at least 1 CL observed by transrectal ultrasonography on day 7 after mating. The present finding is higher than previous study (Gonzalez de Bulnes et al., 1999a) which only recorded 89.3% ovulation rate. These treatments have met our goal to induce multiple ovulation by giving the minimum of 600 IU eCG dosage. Some of the does from G1 (2 does), G2 (3 does) and G3 (3 does) failed to display oestrus within 48 h after CIDR removal. This might be due to either inadequate oestradiol secretion, or oestrus was displayed silently without any overt signs of oestrus (Romano and Wheaton, 1998; Cardwell et al., 1998). Besides that, the absence of oestrus and ovulation may be due to insufficient gonado-trophic hormone released by the pituitary, leading to poor response by the ovary to the exogenous eCG.

Previous studies had reported that the lower pregnancy rate was recorded after a long-term (12 days) progestagen treatment and related to a slower follicular turnover, promoting the ovulation of persistent dominant follicles. In

agreement with that, our findings showed that only 52.4% out of 21 estrus does became pregnant after undergoing 18 days CIDR treatment with eCG treatment on day 17. Our findings were also supported by previous findings of Barrett et al. (2004) who reported that the administration of 500 IU eCG, 12 days after progestagen treatment, had limited effects on the dynamics of ovarian follicular wave development. The short-term progestagen treatment (six days) on the other hand resulted in a higher pregnancy rate, probably due to the ovulation of newly recruited growing follicles (Vinoles et al., 2001).

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Inheritance of grain yield and its correlation with yield components in bread wheat (*Triticum aestivum* L.)

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The genetic effect of grain yield plant⁻¹ and its correlation with yield components were investigated in a 7 × 7 incomplete diallel cross of seven wheat parents during the crop season of 2009 to 2010. Mean square of general combining ability (GCA) effect was 2.90 for grain yield plant⁻¹, which was highly significant (P<0.01), indicating that additive effect played important role in the inheritance of the trait. Specific combining ability (SCA) effect was highly significant (P<0.01) for grain yield plant⁻¹ (0.68), suggesting that the trait was also controlled by non-additive effect. The estimates of GCA showed that the best combiner for grain yield plant⁻¹ was Ningmai 9. The additive-dominance model was partially adequate for grain yield plant⁻¹ and it was controlled by the over dominance type of gene action. Ningmai 8 possessed maximum dominant genes, whereas Yangmai 9 had maximum recessive genes. Grain yield plant⁻¹ might be controlled by two groups of genes and exhibited moderately high value of narrow sense heritability (h²_N=69.51%). The statistical analysis revealed that grain yield plant⁻¹ was positively and significantly correlated with tillers plant⁻¹ (r_p=0.584, r_g=0.595) and number of grains spike⁻¹ (r_p=0.528, r_g=0.507) at phenotypic and genotypic levels. The information obtained from the study might be helpful for wheat breeders trying to develop new varieties with high-yielding potential.

Key words: Combining ability, diallel cross, grain yield, inheritance, *Triticum aestivum* L.

INTRODUCTION

Wheat (*Triticum aestivum* L.) is an important cereal crop next to rice in both acreage and production constituting about 22% of the staple food in China (Lu et al., 2010). Wheat has been cultivated in an area of 24.2 million hectares with the total production of 115 million metric tons in the year of 2010 to 2011 (Shen, 2012). The average yield of wheat in China is 4.75 t ha⁻¹, which is low compared to other leading wheat producers in the world like Germany and France where average yields are 7.4 t and 7.2 t /ha, respectively. The yield is generally insufficient to fulfill the domestic requirements due to the

increase in population (Xiao, 2006). Therefore, it is necessary to develop the new wheat cultivars, having wider genetic base capable of producing better yield under a wide range of agro-climatic conditions to enhance the grain. The grain yield of wheat is determined by three yield components: productive spikes per unit area, number of grains spike⁻¹ and 1000-grain weight (Tian et al., 2012). The grain yield and its components are controlled by many genes, whose expression is greatly influenced by the varying environments (Groos et al., 2003). In most of the diallel studies of wheat, grain

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Table 1. Genetic background and yield traits of the seven parents.

Parent	Pedigree	Released year	Grain yield plant ⁻¹ (g)	Tillers plant ⁻¹	Number of grains spike ⁻¹	1000-grain weight (g)
Ningmai 8	Yangmai 5/Yang 86-17	1996	7.43	4.53	51.17	32.51
Ningmai 9	Yang 86-17/Xifeng	1997	9.03	4.97	55.52	32.86
Yangmai 158	Yangmai 4/st1472-506	1993	9.39	4.80	48.96	39.75
Yangmai 9	Jian 3/Yangmai 5	1996	7.80	4.77	47.98	34.12
Jimai 17	Lingfen 5064/Lumai 13	1999	7.12	5.13	39.40	35.70
Zheng 9023	[83(2)3-3/84(14)43//Xiaoyan 6/Xinong 65]F3/3/Shannong 13	2001	8.05	4.93	37.81	43.45
Yumai 18	Zhengzhou 761/Yanshi 4	1990	7.17	4.93	42.43	34.72

yield plant⁻¹ seemed to be controlled by over dominance type of gene action (Singh and Sharma, 1976; Hussain et al., 2008; Akram et al., 2009; Ojaghi and Akhundova, 2010; Ahmad et al., 2011). However, researchers like Riaz and Chowdhry (2003), Samiullah et al. (2010) and Farooq et al. (2010) observed partial dominance with additive type of gene action for grain yield plant⁻¹. Zhang and Xu (1997) reported that grain yield plant⁻¹ could result from additive and dominant genes with the possibility of epistatic genetic effects. Heritability estimate is a valuable parameter for determining the magnitude of genetic gain from selection. Low, medium, and high narrow sense heritability estimates were reported for grain yield plant⁻¹ (Mckendry et al., 1998; Novoselovic et al., 2004; Liu and Wei, 2006; Erkul et al., 2010; Ahmad et al., 2011). Grain yield plant⁻¹ is a complex trait including number of fertile tillers plant⁻¹, number of grains spike⁻¹ and 1000-grain weight, and it is closely associated with its components (Khaliq et al., 2004; Munir et al., 2007; Ali et al., 2008). The objective of this study was to investigate combining ability, and gene action for grain yield plant⁻¹, and its correlations with yield components in a 7x7 incomplete diallel cross experiment in wheat. This study can be of

great importance in the selection of desirable parents for an effective breeding program to develop the new wheat varieties with high yield potential.

MATERIALS AND METHODS

Plant materials

The experimental material comprised seven wheat varieties: Ningmai 8, Ningmai 9, Yangmai 158, Yangmai 9, Jimai 17, Zheng 9023, and Yumai 18. The parents were chosen based on their broad genetic background and large variations for grain yield plant⁻¹ and yield components (Table 1). These genotypes were crossed in an incomplete diallel fashion during April, 2009. For each of the cross, 15 spikes were emasculated and bagged to avoid contamination with foreign pollen. Pollination with the pollen collected from the specific male parent was done in the morning when the ovaries became receptive. At maturity, the seeds from each cross were harvested and stored separately.

Experimental design

The seeds of seven parents and 21 F₁ hybrids were sown in the field area of Jiangsu Academy of Agricultural Sciences, Nanjing, China, in the first week of November in 2009 and tested in a randomized complete block design with three blocks. Plant-to-plant and row-to-row spacing

was 6.5 and 25 cm, respectively. Within each block, each genotype occupied a plot of two rows of 2 m-long. All the other cultural operations including weeding, fertilizers and disease control were carried out uniformly. At maturity, in late May 2010, ten plants were randomly selected from each of the parents and F₁ progeny to determine fertile tillers plant⁻¹, number of grains spike⁻¹, 1000-grain weight and grain yield plant⁻¹.

Statistical analysis

To assess the differences among parents and F₁ progeny, the data were subjected to analysis of variance using the SAS software. The general and specific combining ability values were estimated using Method II, Model I of Griffing (1956). Two scaling tests (Mather and Jinks, 1982) were applied to test the validity of the additive-dominance model. Further, the Hayman (1954) method was used for estimation of gene actions. Correlation coefficients between grain yield plant⁻¹ and yield components were determined using the data processing system (Tang and Feng, 2002).

RESULTS AND DISCUSSION

Analysis of variance

Analysis of variance revealed highly significant differences ($P \leq 0.01$) among the genotypes for grain yield plant⁻¹ (Table 2). These results

Table 2. Analysis of variance for combining ability for grain yield plant⁻¹.

Source of variation	Degree of freedom	Sum of square	Mean of square	F
Replications	2	7.329	3.664	4.339*
Genotypes	27	95.131	3.523	4.172**
Error	54	45.609	0.845	
GCA	6	17.424	2.904	10.315**
SCA	21	14.287	0.680	2.416**
Error	54	15.203	0.282	

*, ** Significance at P≤0.05 and P≤0.01 levels, respectively.

Table 3. Mean grain yield plant⁻¹ and general combining ability (GCA) effects.

Parent	GCA	Mean (g)
Ningmai 9	0.915 ^{aA}	9.031 ^{aA}
Yangmai 158	0.480 ^{abAB}	9.387 ^{aA}
Zheng 9023	0.143 ^{bcBC}	8.045 ^{bcABC}
Yangmai 9	0.006 ^{bcBCD}	7.799 ^{cBC}
Jimai 17	- 0.325 ^{cdCD}	7.123 ^{cC}
Yumai 18	- 0.594 ^{dD}	7.169 ^{cC}
Ningmai 8	- 0.625 ^{dD}	7.434 ^{cC}
r (GCA, mean)	0.900**	

The values followed by different capital or small letters within the same column are significantly different at the 0.01 and 0.05 probability levels, respectively. ** Significance at P≤0.01 level. r correlation coefficient.

permitted further analysis of combining abilities. Both general combining ability (GCA) and specific combining ability (SCA) variances were highly significant for grain yield plant⁻¹ (Table 2), indicating the importance of both additive and non-additive gene effects. These results are in agreement with earlier findings (Wang et al., 2003; Farooq et al., 2006; Hussain et al., 2012). However, Akram et al. (2011) illustrated that the additive effects were more important for the genetic control of grain yield plant⁻¹.

Performance of parents and combining ability

Mean grain yield plant⁻¹ and GCA effects of the parents are given in Table 3. Significant differences were found for grain yield plant⁻¹ among parents. Yangmai 158 and Ningmai 9 had significant higher grain yield plant⁻¹ than the other five parents and they can be considered as high yielding parents. Zheng 9023 had moderately high yield capacity while Ningmai 8, Yangmai 9, Jimai 17 and Yumai 18 had medium and low yield capacity. Estimates of GCA effects of parents ranged from - 0.625 to 0.915 (Table 3). The highest positive-valued GCA was exhibited in Ningmai 9 followed by Yangmai 158. The GCA value of Ningmai 9 was significantly higher than that of Zheng

9023, Yangmai 9, Jimai 17, Yumai 18 and Ningmai 8 except Yangmai 158. This result indicate that Ningmai 9 was the best combiner for grain yield plant⁻¹ and may serve as genetic sources in breeding programs for increased grain yield. The highest negative-valued GCA was shown in Ningmai 8 followed by Yumai 18. The positive and significant correlation ($r = 0.900$, $P \leq 0.01$) between GCA and parental performance (Table 3) suggested that selection of parents for grain yield plant⁻¹ could be made on the basis of their performance *per se*. The high grain yield plant⁻¹ of certain crosses (Ningmai 8 × Jimai 17, Ningmai 9 × Zheng 9023, and Ningmai 9 × Yangmai9) showed strong positive SCA effects (Table 4). Because in most cases at least one good combining parent was included in these crosses, their progenies had higher grain yield plant⁻¹ than the overall means and yielded desirable transgressive segregations.

Assessment of grain yield plant⁻¹ for additive-dominance model

The data were assessed for additive-dominance (AD) model by exploiting various adequacy parameters given in Table 5. According to Mather and Jinks (1982), the data will be only valid for genetic interpretation if the value of

Table 4. Effects of specific combining ability for grain yield plant⁻¹ in 21 crosses.

Parent	Ningmai 9	Yangmai 158	Yangmai 9	Jimai 17	Zheng 9023	Yumai 18
Ningmai 8	- 0.267	- 0.843	- 0.439	1.377	0.274	0.052
Ningmai 9		0.641	0.999	- 0.152	1.023	0.877
Yangmai 158			0.701	0.664	- 0.693	0.196
Yangmai 9				0.132	0.733	- 0.176
Jimai 17					0.381	- 0.424
Zheng 9023						0.287

$S.E.(\hat{S}_{ij}-\hat{S}_{ik})$: Standard error of differences for SCA effect among crosses with a communal parent; $S.E.(\hat{S}_{ij}-\hat{S}_{kl})$: Standard error of differences for SCA effect among crosses without a communal parent. $S.E.(\hat{S}_{ij}-\hat{S}_{ik}) = 0.708$, $LSD_{0.05} = 1.415$, $LSD_{0.01} = 1.882$; $S.E.(\hat{S}_{ij}-\hat{S}_{kl}) = 0.662$, $LSD_{0.05} = 1.324$, $LSD_{0.01} = 1.760$.

Table 5. Adequacy test of additive-dominance model for grain yield plant⁻¹.

Parameter	Grain yield plant ⁻¹
Joint regression (b)	0.963±0.253
Test for b=0	3.806**
Test for b=1	0.146 NS
Mean squares of Wr + Vr between arrays	1.334 NS
Mean squares of Wr - Vr between arrays	0.230 NS
Fitness of the data to Additive-Dominance model	Partial

** Significance at P≤0.01 level, NS=non significant.

regression coefficient (b) must deviate significantly from zero but not from the unity. The regression analysis revealed that regression coefficient for grain yield plant⁻¹ departed significantly from zero but not from unity, suggesting the absence of non-allelic interactions in genetic behavior of grain yield plant⁻¹ which in turn attested the data valid for AD model for the trait. The appropriateness of the model data analysis was also verified by the analysis of variance of (Wr + Vr) and (Wr - Vr). The lack of significant variation in the (Wr - Vr) arrays suggested that any kind of epistasis was not involved in the phenotypic expression of the trait. Although the value of regression coefficient (b) proved the fitness of the data of grain yield plant⁻¹ for AD model, mean square value of (Wr + Vr) for the trait indicated no significant deviation, thus emphasizing partial validity of the trait. This was also confirmed by Ahmad et al. (2011), Farooq et al. (2011) and Nazeer et al. (2011). The partially adequate model for grain yield plant⁻¹ may be due to the presence of non-allelic interaction, linkage and non-independent distribution of the genes in the parents as suggested by Mather and Jinks (1982).

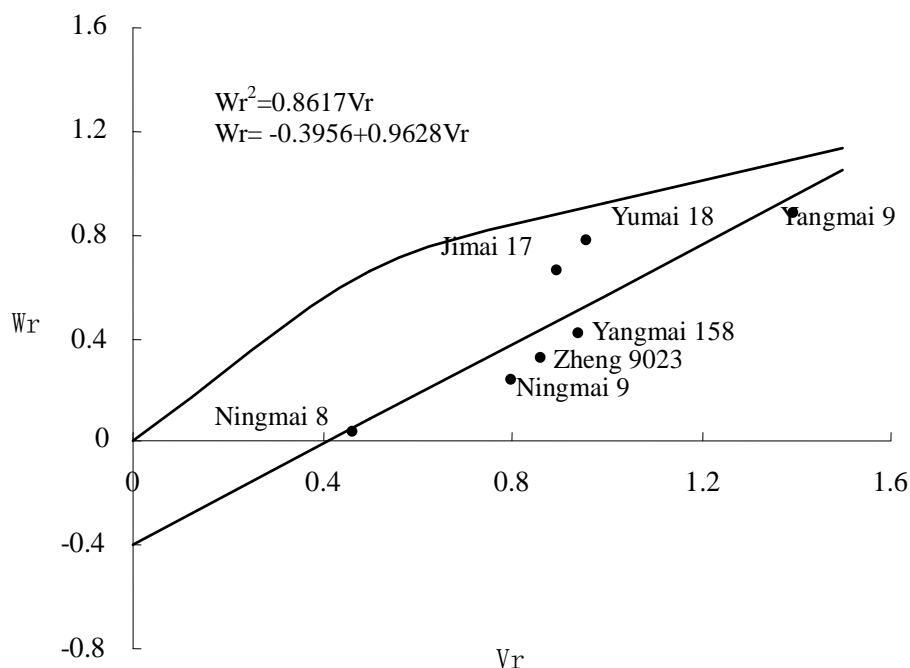
Genetic components of variation for grain yield plant⁻¹

Genetics of grain yield plant⁻¹ was evaluated by calculation of the genetic components of variation D, H₁, H₂ and F (Table 6). Additive (D) and non-additive (H₁ and

H₂) components were significant, indicating that both additive and dominance effects were important components of genetic variation for grain yield plant⁻¹. However, dominance (H₁ and H₂) effects were greater than additive (D), suggesting that non-additive gene action played a predominant role in controlling the genetic mechanism of the trait. The (H₁/D)^{0.5} was more than unity which confirmed the greater contribution of non-additive genes in the inheritance of grain yield plant⁻¹. These results are in accordance with those of Arshad and Chowdhry (2003), Hussain et al. (2008), Akram et al. (2009), Nazeer et al. (2010) and Ojaghi and Akhundova (2010). Preponderance of dominance effects for grain yield plant⁻¹ suggested that the selection for the trait in early generations may not be useful and it had to be delayed till late segregating generations. Asymmetrical distribution of dominant genes was confirmed by unequal estimates of H₁ and H₂, which was further supported by the value of H₂/4H₁ (0.219). The F value, which estimates the relative frequency of dominant to recessive alleles in the parents, was negative. This suggests the excess of recessive alleles present in the parents, which was further supported by the small value (< 1.0) of [(4DH₁)^{0.5}+F]/(4DH₁)^{0.5}-F]. The mean dominance effect of the heterozygote locus (h²) was significant, suggesting that heterosis breeding could be rewarding for this trait. Significant environmental component (E) indicated that the trait was highly affected by environmental conditions (Ahmad et al., 2011). The number of gene groups

Table 6. Estimate of genetic parameters of grain yield plant⁻¹ in a 7×7 diallel cross of wheat.

Genetic parameter	Grain yield plant ⁻¹
D	0.487±0.118**
F	- 0.742±0.282**
H ₁	1.663±0.283**
H ₂	1.455±0.250**
h ²	2.886±0.168**
E	0.315±0.042**
(H ₁ /D) ^{1/2}	1.848
H ₂ /4H ₁	0.219
[(4DH ₁) ^{1/2} +F/((4DH ₁) ^{1/2} -F)	0.416
K	1.984
h ² _N (%)	69.51
R[(W _r +V _r), Pr]	- 0.171

**Figure 1.** Vr versus Wr graph for grain yield plant⁻¹.

differentiating the parents (*k*) was 1.984, suggesting that the inheritance of grain yield plant⁻¹ was controlled approximately by two groups of genes (Dere and Yildirim, 2006; Nazeer et al., 2010). Estimates of narrow sense heritability (*h*²_N) were moderately high for grain yield plant⁻¹. Such moderately high heritable value for grain yield plant⁻¹ was also reported by other researchers (Novoselovic et al., 2004; Yang and Cao, 2005; Ajmal et al., 2009; Akram et al., 2009; Farooq et al., 2010). However, Mckendry et al. (1998), Liu and Wei (2006), Erkul et al. (2010) and Ojaghi and Akhundova (2010) found that the narrow sense heritability for grain yield

plant⁻¹ was low. Differences in the genetic material and analytical technique used in this study could account for these differences.

Graphical (Vr/Wr) representation for grain yield plant⁻¹

The Vr/Wr graph (Figure 1) showed that the regression line intercepted the Wr-axis below the point of origin, suggesting that the trait was controlled by the over dominance type of gene actions and this was superbly maintained by the results provided by the higher values

Table 7. Phenotypic (r_p) and genotypic (r_g) correlation coefficient between grain yield plant⁻¹ and yield components.

Character	r	Tillers plant ⁻¹	Number of grains spike ⁻¹	1000-grain weight
grain yield plant ⁻¹	r_p	0.584**	0.528**	0.251
	r_g	0.595**	0.507**	0.261
Tillers plant ⁻¹	r_p		0.107	- 0.062
	r_g		0.132	0.002
Number of grains spike ⁻¹	r_p			- 0.552**
	r_g			- 0.614**

** Significance at $P \leq 0.01$ levels.

of dominance components H_1 and H_2 over the additive one D. Similar results have earlier been reported by Asif et al. (1999), Chowdhry et al. (2002), Arshad and Chowdhry (2003), Kashif and Khaliq (2003) and Saleem et al. (2005). However, these results were not in accordance with Riaz and Chowdhry (2003) and Samiullah et al. (2010). The distribution of array points along the regression line (Figure 1) revealed that parent Ningmai 8 contained maximum dominant genes being closest to the origin while parent Yangmai 9 carried maximum recessive genes being farthest from the origin. The other two parents Jimai 17 and Yumai 18 also had relatively high frequency of recessive genes. Negative r -value ($r = -0.171$) between parental values (Pr) with ($Wr + Vr$) indicated that the parents with high grain yield plant⁻¹ may carry dominant genes. Similar results were reported by Inamullah et al. (2006) and Ojaghi and Akhundova (2010) while Dere and Yildirim (2006) reported that the parents with high grain yield plant⁻¹ may carry recessive genes.

Correlation analysis

Correlation coefficients between grain yield plant⁻¹ and yield components are shown in Table 7. In general, correlation coefficients at genotypic level were higher than those of phenotypic level. It might be due to the depressing effect of environment on character association as reported earlier for wheat crop (Proda and Joshi, 1970). Grain yield plant⁻¹ had a highly significant positive genotypic correlation with tillers plant⁻¹ ($r = 0.595$, $P \leq 0.01$) and number of grains spike⁻¹ ($r = 0.507$, $P \leq 0.01$), and it showed positive and non-significant correlation with 1000-grain weight at both genotypic and phenotypic levels, suggesting that increase in tillers plant⁻¹ and number of grains spike⁻¹ would increase grain yield plant⁻¹. These results are in agreement with those of Ali et al. (2008). Khaliq et al. (2004) and Munir et al. (2007) reported positive and significant genotypic and phenotypic correlation of grain yield plant⁻¹ with yield

components. Among the yield components, tillers plant⁻¹ was positive and had non-significant correlation with number of grains per spike and 1000-grain weight at genotypic level. Positive and non-significant correlation between tillers plant⁻¹ and number of grains spike⁻¹ at both genotypic and phenotypic levels was also reported by Khan and Dar (2010). Positive and non-significant correlation between tillers plant⁻¹ and 1000-grain weight was found at genotypic level, however the correlation was highly significant (Khokhar et al., 2010), whereas Kashif and Khaliq (2004) reported negative and significant association of tillers plant⁻¹ with 1000-grain weight. There was negative and significant correlation between number of grains spike⁻¹ and 1000-grain weight at both genotypic and phenotypic levels. Similar findings have also been reported by Khan et al. (2010). However, the results were contrary with the findings of Ashfaq et al. (2003).

Conclusion

The results reveal that there was significant genetic variation for grain yield plant⁻¹ among the genotypes. Significant GCA and SCA effects for grain yield plant⁻¹ imply the role of both additive and non-additive gene actions in the genetic control of the trait. Ningmai 9 appeared to be a promising parent for improvement of grain yield plant⁻¹. The additive-dominance model was partially adequate for grain yield plant⁻¹. Dominant genes coupled with moderately high narrow sense heritability were involved in the inheritance of grain yield plant⁻¹, suggesting that selection for the trait in early generations (F_2 - F_3) may not be effective. Grain yield plant⁻¹ had significantly positive genotypic correlation with tillers plant⁻¹ and number of grains spike⁻¹.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Characterization and quantification of phenolic compounds of extra-virgin olive oils according to their blending proportions

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Chemlali olive oil was blended with oils obtained from Oueslati and Chetoui varieties to improve the quality of the former one. Parameters such as triacylglycerols and phenolic compounds were characterized for various blends of Chemlali x Oueslati and Chemlali x Chetoui. Results show that blended oils had an improved composition as compared to that of Chemlali. In fact, the highest percentage of Oueslati and Chetoui olive oils (60% of blending) could lead to 1,2,3-trioleoylglycerol (OOO) of up to 30.20 and 33.44%, respectively. The amount of aldehydic form of oleuropein aglucon was higher when Chemlali was blended using either 40 or 60% of Chetoui olive oil (from 99.17 to 299.63 and 334.16 mg kg⁻¹).

Key words: Oil blending, Chemlali x Oueslati, Chemlali x Chetoui, phenolic fraction, triacylglycerols.

INTRODUCTION

Olive oil is high-value edible oil which is prized for its flavor as well as health characteristics (Harwood and Aparicio, 2000). It is produced predominantly in Southern Europe and North Africa where it forms part of the 'Mediterranean diet' (Harwood and Yaqoob, 2002). Olive oil has a long history and is associated with a low

incidence of coronary-vascular disease (Wahrburg et al., 2002). Its oxidative stability and flavour characteristics are associated with its lipid composition and the presence of minor compounds such as triacylglycerol and phenolic compounds (Baldioli et al., 1996; Servili and Montedoro, 2002). It may also have benefit in reducing obesity, anti-

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Abbreviations: VOO, Virgin olive oil; LLL, 1,2,3-trilinoleylglycerol; LnLO, 1-oleyl-2-linoleyl-3-linolenoylglycerol; LLO, 1,2 dilinoleyl-3-oleylglycerol; LOO, 1,2-dioleoyl-3-linoleylglycerol; OLnO, 1,2-dioleoyl-3-linolenoylglycerol; LnOP, 1-palmitoyl-2-linolenoyl-3-oleylglycerol; LLP, 1-palmitoyl-2,3-dilinoleyl-glycerol; L, 1,2-dioleoyl-3-linoleylglycerol; POL, 1-palmitoyl-2-oleyl-3-linoleylglycerol; OOO, 1,2,3-trioleoylglycerol; POO, 1-palmitoyl-2,3-dioleoyl-glycerol; SOO, 1-stearoyl-2,3-dioleoyl-glycerol; SOP, 1-stearoyl-2-palmitoyl-3-oleylglycerol; AOO, 1-arachidoyl-2,3-dioleoyl-glycerol.

allergic, antimicrobial, anticarcinogenic, antiviral, cancer and inflammatory diseases (Cavaliere et al., 2008; Xia et al., 2008).

Phenolic acids have been associated with colour and sensory qualities, as well as with the health-related and antioxidant properties of foods (Cartoni et al., 2000). One impetus for analytical investigations has been the role of phenolics in the organoleptic properties (flavour, astringency and hardness) of foods (Suárez et al., 2008). Additionally, the content and profile of phenolic acids, their effect on fruit maturation, prevention of enzymatic browning, and their roles as food preservatives has been evaluated (Wu et al., 2008). Several authors have reported that flavonoids such as luteolin and apigenin are also phenolic components of VOO (Carrasco-Pancorbo et al., 2005). Luteolin may originate from rutin or luteolin-7-glucoside, and apigenin from apigenin glucosides. There are also several interesting studies in which several flavonoids have been found in olive leaves and fruits (Kalua et al., 2006).

In Tunisia, the second VOO exporter and producer after the European Union (Haddada et al., 2007a), the two main cultivars are Chemlali and Chetoui. Chetoui, the second main variety cultivated in Tunisia is widespread in the north of the country, occurring in plains as well as in mountain regions, and shows a high capacity of adaptation to various pedo-climatic conditions (Ben Temime et al., 2006). In addition, this oil has a very balanced fatty acid profile and significant/high amount of phenols, which make it quite astringent. This latter characteristic is not accepted by the majority of consumers, especially by kids. Chemlali variety, which is mainly cultivated in the central and southern areas of the country, contributes to 80% of the national olive oil production. It is a productive variety, well adapted to severe environmental conditions. However, its oil is characterized by relatively low levels of oleic acid (54 to 60%), triacylglycerols (Bachir et al., 2007) and phenols (Youssef et al., 2011a). Oueslati variety cultivated in Tunisia is widespread in the centre of the country known by their higher phenol content, >500 mg/kg, stability was measured at 101.6°C, >55 h, oleic acid, >70% and richest on volatile compounds (Youssef et al., 2011b).

Several studies have been published on the analysis of olive oil triacylglycerols (Paradiso et al., 2010; Haddada et al., 2007b; Sánchez et al., 2004) and phenolic compounds (Baccouri et al., 2008; Allalout et al., 2009; Ocakoglu et al., 2009; Youssef et al., 2011a; Stefanoudaki et al., 2011). Many publications reported on the composition of phenolic compounds and triacylglycerols of monovarietal oils, but there are no similar studies on the phenolic compounds and triacylglycerols of oils obtained by blending of monovarietal oils.

The aim of this study was to investigate the effect of blending Chemlali olive oil with the oil obtained from two Tunisian varieties (Oueslati and Chetoui) on phenolic compounds and triacylglycerols.

MATERIALS AND METHODS

Oil samples

Oil was extracted from 3 kg of fruits of each variety during the crop season 2009/2010 (October). Samples were prepared by blending oils of three different cultivars (Chemlali, Oueslati and Chetoui) in different pre-established proportions (20, 40 and 60%) (Youssef et al., 2011b). The olives were washed and deleafed, crushed with a hammer crusher, and the paste was mixed at 25°C for 30 min, centrifuged without addition of warm water and then transferred into dark glass bottles and stored (the olive oils were blended before storage) in the dark at 4°C (1 week) until analysis. Three replicates were prepared for all the samples.

Analysis of total phenolic content

Total phenol and o-diphenol contents were quantified colorimetrically (Ranalli et al., 1999). Phenolic compounds were isolated by triple extraction of a solution of oil (10 g) in hexane (20 ml) with 30 ml of a methanol-water mixture (60:40, v/v). The Folin-Ciocalteu reagent (Merck Schuchardt OHG, Hohenbrunn, Germany) was added to a suitable aliquot of the combined extracts, and the absorption of the solution at 725 nm was measured. Values are expressed as milligrams of caffeic acid per kilogram of oil (Gutfinger, 1981). O-Diphenols were also measured colorimetrically at 370 nm after adding 5% (w/v) sodium molybdate in 50% ethanol to the extract (Gutfinger, 1981). Results are expressed as milligrams of caffeic acid per kilogram of oil.

Analysis of the composition of TAG

Solid phase extraction

The optimized procedure was as follows. VOO (0.2 g) was weighed and dissolved in 0.5 ml *n*-hexane. The silica cartridge (Sep-Pak cartridge, Waters Corporation, USA) was conditioned with 10 ml of *n*-hexane before the application of oil solution. The TAG fraction was obtained with subsequent elution using mixtures of 15 ml of *n*-hexane/diethyl ether (90:10, v/v), and then, the solvent of the collected fractions were evaporated to dryness.

HPLC analysis of triacylglycerol

Extracted TAG (0.05 g) was dissolved in 1 mL of acetone for HPLC analysis, and the injected volume was 0.2 µL. A Hewlett-Packard HPLC (HP 1050, Agilent Technology) quaternary pump instrument equipped with a refractometer detector was employed using a Lichrosorb RP18 column (250 × 4.6 mm, 5 µL particle size; Teknocruma, Barcelona, Spain). Settings were column oven, 45°C; elution solvent, acetone/acetonitrile (70:30, v/v) at a flow rate of 1.4 mL/min for 55 min. The standards used were trilinolein (LLL), triolein (OOO), tripalmitin (PPP), tristearin (SSS), trilinolenin (LnLnLn) and tripalmitolein (PoPoPo) (purity greater than 98%), purchased from Sigma (St. Louis, MO). The abbreviations used for the fatty acids were Po for palmitoleic, L for linoleic, Ln for linolenic, O for oleic, P for palmitic, S for stearic and A for arachidic. TAGs in olive oils were separated according to equivalent carbon number. Determination of the difference between the theoretical value of triacylglycerols (TAGs) with an equivalent carbon number of 42 (ECN 42theoretical) was calculated from the fatty acid composition (Youssef et al., 2011b), and the analytical results (ECN 42HPLC) was obtained by determination of the oil by high performance liquid chromatography.

Analysis of phenolic compounds by RP-HPLC

Analysis of phenolic compounds was carried out by reversed-phase liquid chromatography with ultraviolet detection (Mateos et al., 2001). A solution of p-hydroxyphenyl acetic (0.12 mg/ml) and o-coumaric acids (0.01 mg/ml) in methanol was used as standard. A sample of filtered virgin olive oil (2.5 ± 0.001 g) was weighed and 0.5 ml of standard solution was added. The solvent was evaporated in a rotary evaporator at 40°C under vacuum and the residue was dissolved in 6 ml of hexane. A diol-bonded phase cartridge was placed in a vacuum elution apparatus and conditioned by the consecutive addition of 6 ml of methanol and 6 ml of hexane. The vacuum was then released to prevent drying of the column. The oil solution was applied to the column, and the solvent was pulled through, leaving the sample and the standard on the solid phase. The sample container was washed with 6 ml of hexane, which was run out of the cartridge. The sample container was washed again with 4 ml of hexane/ethyl acetate (85:15, v/v), which was run out of the cartridge and discarded. Finally, the column was eluted with 10 ml of methanol and the solvent was evaporated in a rotary evaporator at room temperature and low speed under vacuum until dryness. The residue was extracted with 0.5 ml of methanol/water (1:1 v/v) at 40°C and the obtained solution was left to rest for 4 h.

Then, the aliquot of 20 μ l of the final solution was injected to the HPLC system. A JASCO HPLC system was equipped with a double plunger pump and a diode array UV detector. A Lichrospher 100 RP-18 column (250 \times 4 mm id, particle size 5 μ m, Merck) was used. The mobile phase consisted of a mixture of water/phosphoric acid (99.5:0.5, v/v) (solvent A) and methanol/acetonitrile (50:50, v/v) (solvent B). The gradient elution program used: 95% (A) / 5% (B) in 0 min 70% (A) / 30% (B) in 25 min 62% (A) / 38% (B) in 40 min 45% (A) / 45% (B) in 45 min 52.5% (A) / 47.5% (B) in 5 min 100% (B) in 5 min 100% (B) was maintained for 5 min and run was ended. Quantification of total phenols was carried out using p-hydroxyphenyl acetic acid as internal standard and the quantification of flavones and ferulic acid was done using o-coumaric acid as internal standard, and the results was expressed in mg kg⁻¹ oil. Triplicate determinations were made.

Statistics

All parameters were determined in triplicate for each sample. Analysis of variance (ANOVA) was conducted using SPSS statistical package (Version 12.0 for Window, SPSS Inc. Chicago, Illinois, 2003). Statistical significance was contrasted using one way ANOVA and Tukey's HSD test at 5% confidence level.

RESULTS AND DISCUSSION

Effect of the mixture of oils in its triacylglycerol composition

Table 1 shows the percentage of all compounds from the triacylglycerol for the various blends obtained from different proportions of two monovarietal oils (Chemlali x Oueslati and Chemlali x Chetoui). The main triacylglycerols detected were 1,2,3-trioleoylglycerol (OOO), 2,3-dioleoyl-1-palmitoylglycerol (POO) and 2,3-dioleoyl-1-linoleoylglycerol (LOO). Other minor triacylglycerols identified were 2,3-dioleoyl-1-stearoylglycerol (SOO), 2-oleoyl-3-palmitoyl-1-stearoylglycerol (SOP), 1-linolenoyl-2-oleoyl-3-palmitoylglycerol (LnOP), 1,2-dilinoleoyl-3-palmitoylgly-

cerol (LLP), 1,3-dioleoyl-2-linolenoylglycerol (OLnO), 1-linolenoyl-2-linoleoyl-3-oleoylglycerol (LnLO) and 1,2,3-trilinoleoylglycerol (LLL) that remain unmentioned. These results are in agreement with the findings of other authors (Bachir et al., 2007). OOO is always the main abundant compound in olive oils, ranging from 24.95 to 38.23% of total triacylglycerols. It was observed that blending could increase OOO (Table 1). At 40% blending with Oueslati and Chetoui olive oil, OOO increased from 24.95 to 30.14 and 32.39%, respectively (Table 1). The blended oils exhibited a significant increase of LOO at the different proportions of mixing oils (from 20 to 60% of blending); in contrast, the 1-palmitoyl-2-oleoyl-3-linoleoylglycerol (POL) and POO decreased (Table 1).

Triacylglycerol composition can also be used as a measurement of the quality and purity of vegetable oils. Elevated levels of trilinolein in olive oil may be used as an indication of the presence of seed oils. However, some olive oils naturally have high trilinolein levels; so, it is more useful to compare the theoretical equivalent carbon number (ECN) for trilinolein, calculated from the fatty acid composition, with the ECN which is determined by analysis. The ECN is defined as $CN-2n$ where CN is the carbon atom number of the fatty acids in the triglycerol molecule and 'n' is the number of double bonds and for trilinolein, it is 42. The difference between the empirical and theoretical ECN42 triacylglycerol content ($\Delta ECN42$) is an European Union official method since 1997. The higher mean value of $\Delta ECN42$ of Chemlali olive oil experienced a significant decrease when Oueslati and Chetoui olive oils were added.

As shown in Table 1, blending of cultivars had significant influence on the other triacylglycerols fractions. These results are consistent with other research in which mixtures of olive oil with other oils from seed sources were investigated on the basis of their triacylglycerol composition (Peter, 1993; Ali et al., 1995).

Effect of the mixture of oils on its phenolic composition

Table 2 shows the concentrations of all the phenolic compounds for the various blends obtained from different proportions of two monovarietal oils (Chemlali x Oueslati and Chemlali x Chetoui).

The analysis of phenolic compounds using reverse phase-high performance liquid chromatography (RPHPLC) with UV detection as described by Mateos et al. (2001), allowed the separation and the identification of these compounds. Results show no qualitative differences in the RPHPLC phenolic fraction profile between virgin olive oils from different proportions. However, significant quantitative differences were observed in a wide number of phenolic compounds.

Chemlali olive oil had poorest level of phenolic compounds in comparison with Chetoui and Oueslati ones. The most important secoiridoids detected were:

Table 1. Triacylglycerols (%) of blended oils in different proportions (Chemlali x Oueslati and Chemlali x Chetoui).

Parameter	Chemlali x Oueslati				Chemlali x Chetoui				
	Chemlali (100%)	Oueslati (20%)	Oueslati (40%)	Oueslati (60%)	Oueslati (100%)	Chetoui (20%)	Chetoui (40%)	Chetoui (60%)	Chetoui (100%)
LLL (%)	0.51 ^a ±0.01	0.52 ^a ±0.01	0.50 ^b ±0.00	0.49 ^b ±0.03	0.46 ^c ±0.01	0.48 ^b ±0.05	0.45 ^c ±0.02	0.41 ^d ±0.03	0.38 ^e ±0.08
LnLO (%)	0.30 ^a ±0.02	0.32 ^b ±0.02	0.36 ^c ±0.03	0.37 ^d ±0.01	0.48 ^e ±0.06	0.31 ^b ±0.02	0.30 ^c ±0.02	0.32 ^d ±0.03	0.36 ^e ±0.02
LLO (%)	5.99 ^a ±0.11	5.97 ^a ±0.12	6.14 ^{ab} ±0.08	6.29 ^b ±0.05	6.97 ^c ±0.62	5.66 ^b ±0.09	5.17 ^c ±0.05	5.04 ^d ±0.04	4.59 ^e ±0.41
OLnO (%)	1.56 ^a ±0.02	1.42 ^b ±0.04	1.21 ^c ±0.03	1.07 ^d ±0.02	0.84 ^e ±0.03	1.49 ^b ±0.01	1.39 ^c ±0.03	1.16 ^d ±0.06	0.95 ^e ±0.22
LnOP (%)	2.61 ^a ±0.03	2.70 ^a ±0.01	2.06 ^b ±0.01	1.98 ^b ±0.07	1.35 ^c ±0.11	2.33 ^b ±0.04	2.10 ^c ±0.01	1.93 ^d ±0.07	1.10 ^e ±0.02
LLP (%)	1.34 ^a ±0.04	1.29 ^b ±0.02	1.10 ^c ±0.02	0.93 ^d ±0.01	0.38 ^e ±0.06	1.29 ^b ±0.01	1.13 ^c ±0.03	0.89 ^d ±0.01	0.40 ^e ±0.09
LOO (%)	17.10 ^a ±0.14	17.94 ^b ±0.22	18.62 ^c ±0.19	18.99 ^d ±0.44	21.00 ^e ±0.17	17.32 ^b ±0.21	18.89 ^c ±0.17	19.95 ^d ±0.33	21.00 ^e ±0.62
POL (%)	13.83 ^a ±0.21	12.87 ^b ±0.05	11.11 ^c ±0.02	10.86 ^d ±0.51	8.73 ^e ±0.12	12.95 ^b ±0.11	12.06 ^c ±0.11	10.15 ^d ±0.12	8.02 ^e ±0.11
OOO (%)	24.95 ^a ±0.45	25.26 ^b ±0.02	30.14 ^c ±0.03	30.20 ^{cd} ±0.74	34.23 ^e ±1.11	25.69 ^b ±0.53	32.39 ^c ±0.92	33.44 ^d ±0.31	38.23 ^e ±0.19
POO (%)	21.13 ^a ±0.76	21.15 ^a ±0.06	20.06 ^b ±0.55	19.93 ^{bc} ±0.35	17.19 ^d ±0.59	20.77 ^b ±0.77	19.03 ^c ±0.22	18.12 ^d ±0.13	15.10 ^e ±1.02
SOO (%)	5.09 ^a ±0.01	4.89 ^b ±0.03	4.30 ^c ±0.12	3.49 ^d ±0.11	2.90 ^e ±0.01	4.97 ^b ±0.10	3.79 ^c ±0.00	3.32 ^d ±0.03	2.91 ^e ±0.34
SOP (%)	4.61 ^a ±0.02	4.65 ^b ±0.03	4.58 ^c ±0.03	4.51 ^c ±0.10	4.40 ^d ±0.41	4.72 ^b ±0.14	4.44 ^c ±0.04	4.35 ^d ±0.03	3.91 ^e ±0.03
AOO (%)	0.98 ^a ±0.01	1.02 ^a ±0.02	0.92 ^b ±0.01	0.89 ^c ±0.00	0.81 ^d ±0.03	0.97 ^a ±0.00	0.96 ^b ±0.03	0.97 ^b ±0.02	1.00 ^c ±0.05
ECN ₄₂	0.93 ^a ±0.03	0.77 ^b ±0.01	0.049 ^c ±0.00	0.037 ^d ±0.01	0.14 ^e ±0.04	0.64 ^b ±0.02	0.41 ^c ±0.02	0.31 ^d ±0.04	0.20 ^e ±0.01

^{a-e}Mean ± SD, significant differences within the same row are shown by different letters (P < 0.05). Data values expressed in %. LLL, 1,2,3-Trilinoleylglycerol; LnLO, 1-oleyl-2-linoleyl-3-linolenoylglycerol; LLO, 1,2-dilinoleyl-3-oleylglycerol; LOO, 1,2-dioleoyl-3-linoleylglycerol; OLnO, 1,2-dioleoyl-3-linolenoylglycerol; LnOP, 1-palmitoyl-2-linolenoyl-3-oleylglycerol; LLP, 1-palmitoyl-2,3-dilinoleyl-glycerol; L, 1,2-dioleoyl-3-linoleylglycerol; POL, 1-palmitoyl-2-oleyl-3-linoleylglycerol; OOO, 1,2,3-trioleoylglycerol; POO, 1-palmitoyl-2,3-dioleoyl-glycerol; SOO, 1-stearoyl-2,3-dioleoyl-glycerol; SOP, 1-stearoyl-2-palmitoyl-3-oleylglycerol; AOO, 1-arachidoyl-2,3-dioleoyl-glycerol.

Dialde-hydric form of ligostroside aglucon, dialdehydic form of oleuropeine aglucon and aldehydic form of oleuropeine aglucon.

The low dialdehydic form of ligostroside aglucon content (<47.11 mg kg⁻¹) of Chemlali olive oil, experienced a significant increase when Oueslati and Chetoui olive oils were added (Table 2). The amount of dialdehydic form of ligostroside aglucon content slowly increased with the percentage of blending. It is also remarkable that Chetoui olive oil is the best one for dialdehydic form of ligostroside aglucon fortification in Chemlali olive oil (123.27 mg kg⁻¹). For the aldehydic form of oleuropeine aglucon, virgin olive oil from Oueslati showed the lowest value (25.63 mgkg⁻¹) and the

highest value was observed from Chetoui (578.18 mg kg⁻¹). At 60% blending with Chetoui olive oil, aldehydic form of oleuro-peine aglucon increased from 99.17 to 334.16 mg kg⁻¹, and oleuropeine aglucon tyrosol acetate decreased from 5.36 to 2.12 mg kg⁻¹ (Table 2). Using 60% Oueslati olive oil, aldehydic form of oleuropeine aglucon underwent a significant de-crease to 54.88 mg kg⁻¹ and, at the same time, a decrease of oleuropeine aglucon tyrosol acetate to 3.12 mg kg⁻¹ was observed.

With regards to phenolic acids, the concentration of vanillic acid, vanillin, p-coumaric acid and ferulic acid were dissimilar in different blends of olive oil. All the oils produced and analysed (Table

2) showed very low values for the phenolic acid (vanillic, p-coumaric and ferulic acids). These results are similar to those reported by several authors for other olive oil varieties (Krichene et al., 2009; Youssef et al., 2011a). There were only minor changes in the amount of phenolic compounds when olive oil from both cultivars were blended (Table 2).

The main simple phenols found in the Chemlali, Oueslati and Chetoui virgin olive oil were hydroxytyrosol and tyrosol. The concentration of hydroxytyrosol, was generally higher than that of tyrosol. The blending process improved phenols by increasing the hydroxytyrosol and tyrosol content of Chemlali oil. At 60% blending, hydroxytyrosol

Table 2. Phenolic composition (mg.kg⁻¹) of blended oils in different proportions (Chemlali x Oueslati and Chemlali x Chetoui).

Parameter	Chemlali x Oueslati					Chemlali x Chetoui			
	Chemlali (100%)	Oueslati (20%)	Oueslati (40%)	Oueslati (60%)	Oueslati (100%)	Chetoui (20%)	Chetoui (40%)	Chetoui (60%)	Chetoui (100%)
Hydroxytyrosol (mg.kg ⁻¹)	1.74 ^a ±0.02	1.66 ^b ±0.03	1.42 ^c ±0.02	4.04 ^d ±0.00	7.43 ^e ±0.03	3.17 ^b ±0.00	6.87 ^c ±0.03	10.11 ^d ±0.02	19.79 ^e ±0.53
Tyrosol (mg.kg ⁻¹)	1.13 ^a ±0.04	1.31 ^b ±0.05	1.30 ^b ±0.03	1.61 ^c ±0.02	1.94 ^d ±0.03	5.12 ^b ±0.12	8.43 ^c ±0.02	14.17 ^d ±0.13	25.22 ^e ±0.66
Vanillic acid (mg.kg ⁻¹)	tr	0.11 ^b ±0.00	0.11 ^b ±0.01	0.12 ^b ±0.04	0.29 ^c ±0.05	0.09 ^b ±0.00	0.11 ^c ±0.00	0.14 ^d ±0.02	0.28 ^e ±0.02
Vanillin (mg.kg ⁻¹)	0.66 ^a ±0.01	0.65 ^a ±0.12	0.64 ^a ±0.00	0.61 ^b ±0.03	0.50 ^c ±0.01	0.63 ^b ±0.01	0.59 ^c ±0.02	0.48 ^d ±0.01	tr
p-coumaric acid (mg.kg ⁻¹)	tr	0.10 ^b ±0.01	0.19 ^c ±0.01	0.29 ^d ±0.01	0.54 ^e ±0.09	0.14 ^b ±0.02	0.22 ^c ±0.01	0.34 ^d ±0.03	0.85 ^e ±0.02
Ferulic acid (mg.kg ⁻¹)	tr	0.12 ^b ±0.01	0.18 ^c ±0.02	0.27 ^d ±0.02	0.52 ^e ±0.03	0.08 ^b ±0.00	0.11 ^c ±0.02	0.15 ^d ±0.02	0.41 ^e ±0.03
Hydroxytyrosol acetate (mg.kg ⁻¹)	0.03 ^a ±0.00	0.14 ^b ±0.02	0.32 ^c ±0.07	0.63 ^d ±0.02	1.07 ^e ±0.02	0.10 ^a ±0.01	0.16 ^b ±0.00	0.19 ^c ±0.00	0.32 ^d ±0.03
Dialdehydic form of oleuropeine aglucon (mg.kg ⁻¹)	16.32 ^a ±0.52	21.17 ^b ±0.33	28.44 ^c ±0.04	34.17 ^d ±0.54	46.22 ^e ±1.00	20.35 ^a ±0.11	26.18 ^b ±0.23	37.12 ^c ±0.02	53.11 ^d ±0.34
Oleuropeine aglucon tyrosol acetate (mg.kg ⁻¹)	5.36 ^a ±0.12	5.03 ^b ±0.14	4.66 ^c ±0.07	3.12 ^d ±0.21	tr	5.29 ^b ±0.07	4.89 ^c ±0.12	2.12 ^d ±0.01	2.04 ^e ±0.02
Dialdehydic form of ligostroside aglucon (mg.kg ⁻¹)	47.11 ^a ±1.11	60.17 ^b ±1.41	81.28 ^c ±1.37	99.15 ^d ±1.34	128.74 ^e ±1.15	68.68 ^b ±0.89	98.14 ^c ±0.04	123.27 ^d ±0.77	199.22 ^e ±0.98
Aldehydic form of oleuropeine aglucon (mg.kg ⁻¹)	99.17 ^a ±1.46	90.27 ^b ±1.01	70.19 ^c ±1.14	54.88 ^d ±1.21	25.21 ^e ±0.46	133.69 ^f ±1.11	299.63 ^a ±1.23	334.16 ^b ±1.44	578.18 ^c ±1.11
Aldehydic form of ligostroside aglucon (mg.kg ⁻¹)	37.55 ^a ±0.52	33.75 ^b ±0.78	29.28 ^c ±0.67	25.63 ^d ±0.34	14.77 ^e ±0.12	42.55 ^b ±0.56	53.18 ^c ±0.61	71.03 ^d ±0.81	122.16 ^e ±0.13
Pinoresinol (mg.kg ⁻¹)	3.66 ^a ±0.02	3.32 ^a ±0.22	3.10 ^a ±0.03	2.47 ^b ±0.08	tr	3.98 ^b ±0.02	4.14 ^c ±0.06	5.22 ^d ±0.22	7.22 ^e ±0.12
Acetoxypinoresinol (mg.kg ⁻¹)	1.03 ^a ±0.03	4.18 ^b ±0.03	7.98 ^c ±0.11	11.16 ^d ±0.04	19.53 ^e ±0.52	3.13 ^b ±0.04	4.99 ^c ±0.01	6.87 ^d ±0.31	11.54 ^e ±0.21
Luteolin (mg.kg ⁻¹)	1.13 ^a ±0.01	1.13 ^a ±0.02	1.29 ^b ±0.03	1.36 ^c ±0.03	1.66 ^d ±0.01	1.29 ^b ±0.05	1.45 ^c ±0.01	1.53 ^d ±0.01	1.96 ^e ±0.05
Apigenin (mg.kg ⁻¹)	0.43 ^a ±0.03	0.44 ^a ±0.03	0.45 ^b ±0.05	0.41 ^c ±0.02	0.46 ^d ±0.02	0.51 ^b ±0.01	0.62 ^c ±0.02	0.73 ^d ±0.02	1.39 ^e ±0.04
Phenols (mg.kg ⁻¹)	190.09 ^a ±0.4	215.17 ^b ±2.40	283.17 ^c ±1.27	401.2 ^d ±2±1.01	510.42 ^e ±0.40	254.12 ^b ±0.6	406.14 ^c ±0.32	736.89 ^d ±4.66	928.88 ^e ±13.2
o-Diphenols (mg.kg ⁻¹)	91.13 ^a ±0.20	100.65 ^b ±0.16	120.31 ^c ±2.06	141.42 ^d ±0.12	185.62 ^e ±0.20	93.08 ^b ±1.27	142.14 ^c ±2.22	202.19 ^d ±3.47	282.82 ^e ±40.95

^{a-e}Mean ± SD, significant differences within the same row are shown by different letters (P < 0.05). tr: < 0.1%; Data values are expressed in mg.kg⁻¹.

increased from 1.74 to 4.04 and 10.11 mg kg⁻¹, while tyrosol increased from 1.13 to 1.61 and 14.17 mg kg⁻¹ when Oueslati and Chetoui olive oil was used respectively.

Lignans are present also in considerable amount, particularly acetoxypinoresinol which is found in all analysed samples at concentrations ranging from 1.03 mg kg⁻¹ in virgin olive oils from Chemlali to 11.54 and 19.53 mg kg⁻¹ in virgin olive oils from Chetoui and Oueslati, moreover, significant differences were observed in its contents with different proportions (Table 2). The process was observed only when the blending was carried out at important percentages, such as 60% (from 1.03 to 11.16 mg kg⁻¹ with Oueslati and from 1.03 to 6.87 mg kg⁻¹ with Chetoui olive oils), while, pinoresinol

is detected in low concentration not more than 7.22 mg kg⁻¹ (Table 2).

As seen in simple phenols and secoiridoids, a considerable variation in lignans concentrations between olive oils of various proportions also occurs in this case, the reasons probably being related when some cultivars are contemporary milled separately and then obtained oils were blended, some interactions and/or synergisms could occur among the enzymes involved in the lipoxygenase cascade.

Low flavonoid levels represented by luteolin and apigenin were observed in all the olive oils analysed, with concentrations that varied from 1.13 to 1.96 mg kg⁻¹ and 0.43 to 1.39 mg kg⁻¹, respectively.

The low phenol content (<200 mg kg⁻¹) of

Chemlali olive oil showed a significant increase when Oueslati and Chetoui olive oils were added (Table 2). The amount of phenols slowly increased with the percentage of blending. At 20%, the amount of phenols increased from 190.09 to 215.17 and 254.12 mg kg⁻¹ with Oueslati and Chetoui olive oils, respectively. At the highest percentages (60%) with Oueslati, o-diphenols in Chemlali olive oil underwent about twofold increase. Further, 60% of blending with Chetoui oil improved the amount of total phenols in Chemlali from 91.13 to 202.19 mg/kg (threefold). The concentrations of total phenols and o-diphenols appeared to proportionally vary according to the relative proportion of each monovarietal oils in the mixtures.

Conclusion

The changes of quality of monovarietal VOOs play an important role in blending application. The blending process using different percentages of other olive oils improved the fatty acid composition and volatile compounds (Youssef et al., 2011b). The lowest proportions to obtain blends endowed with equilibrated triacylglycerols and phenolic compound were about 40%. In addition, these results confirmed that the accumulation of each triacylglycerols and phenolic compound in monovarietal oils was different and closely dependent on the genetic store of each variety. The understanding of the pathway that produces the phenolic compounds is also important in enhancing the quality of olive oils (Youssef et al., 2011a). Therefore, blending of cultivars had significant influence on the triacylglycerols fractions and phenolic compounds.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Chicken fat and inorganic nitrogen source for lipase production by *Fusarium* sp. (*Gibberella fujikuroi* complex)

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In this work, we report the production of lipases by a *Fusarium* sp. isolate (FCLA-MA41) from Atlantic Forest, using chicken fat and association of organic and inorganic nitrogen sources in submerged fermentation to seek economically attractive bioprocess. A 2-level, 4-factor Central Composite Design (CCD) and response surface methodology (RSM) were used to study the influence of the concentrations of chicken fat, $(\text{NH}_4)_2\text{SO}_4$, Triton X-100 and yeast extract as a nutrient supplement in Vogel minimum salt medium. RSM defined the region with the best response as consisting of the following combination of variables: chicken fat 15.0 mL/L, Triton X-100 15.0 g/L, ammonium sulfate 4.5 g/L and yeast extract 1.0 g/L. A validation study was performed according to the described concentrations and produced an enzymatic activity of 4.22 ± 0.35 U/mL. Considering the cost estimates of the nutrient medium optimized for lipase production, the production cost was \$US 518.00/million Units of lipase.

Key words: Fungal enzyme, central composite design, chicken fat, ammonium sulfate, Triton X-100.

INTRODUCTION

The interest of this work is the triacylglycerol lipases (E.C.3.1.1.3), which are enzymes, described as glycerol ester hydrolases acting on ester bonds present in acylglycerols, releasing fatty acids and glycerol (Jaeger et al., 1994). They constitute a special class among the carboxylic ester hydrolases (Egloff et al., 1995). The use of lipases has increased considerably, especially in the

food, beverage, textile, pharmaceutical, cosmetic, bio-energetics, fine chemicals and pulp and paper industries. Currently, enzymes are produced naturally from plants, animals, fungi, yeasts and bacteria. When microorganisms are used, they can be inoculated in residues resulting from food processing, thereby reducing the production cost.

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Fungi of different genera proven to be good producers of lipases and enzymes have been previously studied (Singh and Mukhopadhyay, 2012). However, the costs of production of microbial lipases have limited the use of these enzymes. The potential of using low-cost nutrients such as agro-industrial residues in microbial fermentation for enzyme production is extremely important in dictating future uses of lipases. One critical factor in producing microbial lipases is the choice of carbon sources as enzyme inducers. Compounds such as plant seed-oils (triacylglycerols), free fatty acids, surfactants, bile salts and glycerol have been included in the nutrient medium to increase levels of lipase activity (Gupta et al., 2004). Corn oil has frequently been cited as an inducer of *Fusarium* lipases (Maia et al., 2001; Rifaat et al., 2010), however, vegetable oils are regarded as expensive fermentation substrates, and are mainly used as a food stock.

Animal fat has the advantage of wide availability and low cost, and waste product of meat processing. Chicken fat is waste product of poultry processing industry (Arnaud et al., 2004) which has been used to produce biodiesel. However, as shown in this work, it can also be used for obtaining bio-products with high added value such as enzymes. The average cost of the disposal is of \$ 0.60/ L, 80% cheaper than olive oil, mostly used substrate for lipase production from microorganisms (Benjamin and Pandey, 2001; Hatzinikolaou et al., 1996; Long et al., 1996) and 50% cheaper than corn oil, which is frequently being cited as an inducer of *Fusarium* lipases (Maia et al., 2001; Rifaat et al., 2010; "USDA Economic Research Service," 2013). To date, no other work in the literature has reported the use chicken fat to induce production of lipases. The chicken fat used in this work was obtained from poultry slaughtering which was discarded as waste. The fatty acid composition of the chicken fat used for this work has 95% of long chain fatty acids and can thus serve as a source of carbon for inducing production of the enzyme.

Although, the carbon source is the key choice for lipase production, due to the inductive effect and also the costs associated with it, the N source occupies a prominent place especially for the consequences in upstream process (Keller et al., 2001). The organic sources often provide higher enzymatic activity in cultures with microorganisms, but are costly in regard to inorganic salts and complicate the purification due to the complex composition that includes proteins and peptides. Thus, studies to minimize or even to avoid their use as a component in the culture medium are encouraged. Although not well studied, lipases from *Fusarium* spp. (filamentous fungi) are known to exhibit some interesting properties, such as their stability in polar organic solvents like ethanol, acetone and n-propanol (Camargo-de-Morais et al., 2003). Recently a strain of *Fusarium* sp. (*Gibberella fujikuroi* complex) FCLA-MA41 (Oliveira et al.,

2013) was isolated from decaying plant matter in the Atlantic Forest of the state of São Paulo in Brazil. Cultivation of the strain in Submerged Fermentation, a medium containing crambe oil (17.5 mL/L), Triton X-100 (5 g/L), ammonium sulfate (5 g/L) and yeast extract (1 g/L) was proposed, resulting in a lipase titer of 3.0 ± 0.25 U/mL. Using Solid State Fermentation, the same fungus produced a maximum lipase titer of 5.0 ± 0.25 U/gds on crambe meal moistened with phosphate buffer.

In this work, we report on the production of lipases by a *Fusarium* sp. (GFC) isolate FCLA-MA41, using chicken fat and association of organic and inorganic nitrogen source in submerged fermentation to seek economically attractive bioprocess.

MATERIALS AND METHODS

Materials

The chicken fat was kindly supplied by Fricock Frigorificação Avicultura Indústria Comércio Ltda. (Rio Claro-SP, Brazil).

Microorganism

The fungal strain *Fusarium* sp. (*Gibberella fujikuroi* complex) FCLA-MA41 was isolated from decaying plant matter in the Atlantic Forest in the state of São Paulo (Brazil) and identified as described by Oliveira et al. (2013). The fungal isolate was maintained on MEA medium (malt extract agar) and stored at 4°C. For spore production, the fungal isolate was grown at 28°C for 5 to 7 days. A spore suspension was prepared at a concentration of 1×10^8 spores/mL and glycerol added to a final concentration of 200 mL/L. Aliquots of this preparation was transferred into cryovials for storage at -20°C. To ensure sterile growth of the fungal isolate, solid medium was autoclaved at 121°C for 20 min and used for propagation, inoculum and production purposes.

Enzyme production by submerged fermentation (SmF)

Optimization of enzyme production in SmF using factorial design

A 2-level, 4-factor Central Composite Design (CCD) and response surface methodology (RSM) were used to study the influence of the concentrations of chicken fat (X_1), $(\text{NH}_4)_2\text{SO}_4$ (X_2), Triton X-100 (X_3) and yeast extract as a nutrient supplement (X_4) in Vogel minimum salt medium (VMSM) (Vogel, 1956). Table 1 shows the independent factors (X_i), their levels and the experimental design in terms of the coded (- α , -1, 0, 1 and + α) and the non-coded (actual value) variables. The analyses were performed using STATISTICA 8.0 software (Statsoft Inc.) to calculate the main effects of the variables and their interactions, and to perform the analysis of variance (ANOVA). The response of variables, Y (lipase activity, U/mL), may be approximated by the polynomial equations:

$$\text{CCD } 2^4: Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j$$

where: Y is the predicted response, β_0 is the offset term, β_i the linear effect, β_{ii} the squared effect and β_{ij} the interaction effect.

The inoculum was prepared by transferring three agar discs (0.25

cm diam.) colonized with mycelium to a 250 mL Erlenmeyer flask containing 50 mL of nutrient medium. The flasks were kept at 28°C and shaken at 180 rpm for 72 h (best fermentation time – data not show). After fermentation, the culture fluid was filtered through gauze cloth, used as the source of extracellular enzyme and the biomass was dried and determined by gravimetry. The tests were carried in triplicate.

Analytical methods

Lipase assay and protein determination

The lipase activity of the fermentation samples was measured by the hydrolysis of *p*-nitrophenyl palmitate (*p*NPP) as first described by Winkler and Stuckmann (1979), and modified by Lima et al. (2004). One unit of lipase activity is defined as the release of 1 mmol/min of *p*-nitrophenol (*p*NP). The molar extinction coefficient of *p*NP (1.5×10^4 mol/L x cm) was used to correlate the concentration of product from the absorbance readings.

Gas chromatography (GC) analysis of fatty acids

The fatty acid derived from chicken fat were identified at Technology Laboratory of Animal Products (UNESP – Jaboticabal/SP) by GC using a Shimadzu 14B Gas Chromatograph connected to a OMEGAWAX250 capillary column (30 m x 0.25 mm x .25 µm). The injector and detector temperatures were set at 250 and 280°C, respectively. The column temperature was initially maintained at 100°C for 2 min, increased to 220°C at 4°C/min, and finally held at 300°C for 25 min. A mix of fatty acids (SIGMA) was used as standard.

RESULTS

Fatty acid composition of chicken fat

The chicken fat oil used in this study was analyzed by GC to assess its fatty acid composition, verifying that it was composed of 24.61% palmitic acid (C-16:0), 9.39% palmitoleic acid (C-16:1), 4.84% stearic acid (C-18:0), 42.93% oleic acid (C-18:1), 13.63% linoleic acid (C-18:2) and 4.60% of others as reported elsewhere (Arnaud et al., 2004).

Effect of concentration of chicken fat, Triton X-100, ammonium sulfate and yeast extract on lipase production using CCD matrix analysis

A 2⁴ CCD matrix was used to evaluate the process variables adopted in this experiment. The variables included the concentration of chicken fat (X_1), Triton X-100 (X_2), ammonium sulfate (X_3) and yeast extract (X_4) in nutrient media containing VMSM. Time (72 h), orbital agitation (180 rpm) and fermentation temperature (28°C) were the fixed parameters in the experimental design. Table 1 presents the experimental matrix with the variables in their coded forms. The responses (lipase activity) are presented

in U/mL. Highest lipase activities were observed in runs 14 and 24 with chicken fat resulting in activities of 8.33 and 8.04 U/mL, respectively.

ANOVA indicated that there was no interaction between the variables at a confidence level of $p < 0.05$. However, when considered in isolation, variables X_2 , X_3 and X_4 produced statistically significant results. Triton X-100 (X_2) and yeast extract (X_4) showed a positive influence on the response, and therefore increasing these variables led to an increase in lipase activity of 1.92 and 2.37 U/mL, respectively. On the other hand, increasing the concentration of ammonium sulfate (X_3) has a negative effect on the response, causing a decrease in lipase activity of 1.59 U/mL.

After testing the validity, the F test, $F_{\text{calc}} > F_{\text{tab}}$ (Table S1), indicated that the model was statistically significant (valid). An empirical mathematical model of lipase production can be described by Equation 1.

$$Y \text{ (U/mL)} = 5.1975 + 0.9600.(X_2) - 0.7975.(X_3) + 1.1841.(X_4) \quad (1)$$

Three-dimensional response surfaces obtained for the model are shown in Figure 1, the contour plots are shown in Figure S1. Thus, RSM defined the region with the best response as consisting of the following combination of variables: chicken fat 15.0 mL/L, Triton X-100 15.0 g/L, ammonium sulfate 4.5 g/L and yeast extract 1.0 g/L. This combination was predicted to produce a lipase activity of 9.11 ± 5.0 U/mL. A validation study was performed according to the described concentrations and produced lower values than expected, but still within the margin of error, with an enzymatic activity of 4.22 ± 0.35 U/mL.

Costs of lipase production by *Fusarium* sp. (GFC) FCLA-MA41

Considering the cost estimates of the nutrient medium optimized for lipase production by SmF containing 15.0 mL/L of chicken fat (\$0.60/ L), 4.5 g/L of ammonium sulfate (\$15.75/ kg), 1.0 g/L of yeast extract (\$173.20/ kg) and 15.0 g/L of Triton X-100 (\$80.00/ kg) in a solution containing Vogel minimum salts (\$0.73/ L) used in this study, the production cost was \$US 518.00/million Units of lipase.

DISCUSSION

Rapp (1995) described the addition of trimyristin, olive oil, oleic acid, and surfactant Span 85 inducing formation of extracellular lipolytic activity in cultures from *Fusarium oxysporum* sp. *vasinfectum*. *F. oxysporum* lipase was induced by rape seed oil (Tamerler and Keshavarz, 2000) and triolein (Camargo-de-Morais et al., 2003); sesame oil

Table 1. CCD matrix (factorial 2⁴) containing four repetitions at the central point for the production of lipase by *Fusarium* sp. (GFC) FCLA-MA-41 in SmF.

Run	Variable in coded levels				Response				
					(Y, U/mL)		Biomass (g/L)	At/Biomass (U/g)	
	X ₁	X ₂	X ₃	X ₄	Predicted	Observed	Observed	Observed	
1	-1	-1	-1	-1	0.00	2.37	11.57	204.84	
2	-1	-1	-1	+1	2.88	3.07	12.05	254.77	
3	-1	-1	+1	-1	1.65	0.93	13.60	68.38	
4	-1	-1	+1	+1	4.28	4.37	11.75	371.91	
5	-1	+1	-1	-1	5.79	4.39	12.53	350.36	
6	-1	+1	-1	+1	8.04	6.87	8.95	767.60	
7	-1	+1	+1	-1	3.84	5.24	13.12	399.39	
8	-1	+1	+1	+1	5.80	5.81	11.44	507.87	
9	+1	-1	-1	-1	2.73	3.18	16.36	194.38	
10	+1	-1	-1	+1	5.51	5.17	17.51	295.26	
11	+1	-1	+1	-1	1.78	4.01	14.64	273.91	
12	+1	-1	+1	+1	4.27	6.13	14.32	428.07	
13	+1	+1	-1	-1	5.05	6.01	11.34	529.98	
14	+1	+1	-1	+1	7.16	8.33	18.27	455.94	
15	+1	+1	+1	-1	0.46	0.72	21.39	33.66	
16	+1	+1	+1	+1	2.27	0.91	20.66	44.05	
17	-α	0	0	0	2.98	3.35	7.53	444.89	
18	+α	0	0	0	2.23	0.39	20.61	18.92	
19	0	-α	0	0	2.45	0.13	18.78	6.92	
20	0	+α	0	0	6.29	7.13	14.56	489.70	
21	0	0	-α	0	4.87	4.50	12.36	364.078	
22	0	0	+α	0	1.68	0.57	20.62	27.64	
23	0	0	0	-α	2.75	0.73	13.90	52.52	
24	0	0	0	+α	7.49	8.04	13.77	583.88	
25 ^a	0	0	0	0	5.19	5.95	14.78	402.57	
26 ^a	0	0	0	0	5.19	4.89	14.23	343.64	
27 ^a	0	0	0	0	5.19	1.80	15.43	116.66	
28 ^a	0	0	0	0	5.19	1.45	15.79	91.83	
Factor					Real level				
					-α	-1	0	+1	+α
X ₁ (Chicken fat, mL/L)					10.0	15.0	20.0	25.0	30.0
X ₂ (Triton X-100, g/L)					0.0	5.0	10.0	15.0	20.0
X ₃ ((NH ₄) ₂ SO ₄ , g/L)					1.0	4.5	10.0	15.5	19.0
X ₄ (Yeast extract, g/L)					0.00	0.25	0.50	0.75	1.00

α = 2.0;^a Central point runs.

was used to induce lipase from *Fusarium solani* (Maia et al., 2001). The chicken fat used in this study, according to analysis, is composed of long-chain fatty acids, primarily oleic (18:1, 42.93%), palmitic (16:0, 24.61%) and linoleic (18:2, 13.63%). With this composition, this food-processing residue could induce lipase production from *Fusarium* sp. (GFC). In fact this was observed, because enzyme activity was not detected in runs conducted in the absence of chicken fat (data not show). When com-

paring the runs 17 and 18, it is clear, the importance of chicken fat for microorganism growth, but the increase in its concentration has little influence on the extracellular lipase activity.

In the literature it is common to justify the reduction of extracellular activity with increasing addition of oil due to the aeration difficulty of the culture medium, which affect cell metabolism (Li et al., 2006). However, even with the adding of surfactant Triton X-100 as a variable in this

Table S1. Analysis of variance of enzymatic activity (U/mL) obtained for the 2⁴CCD matrix with chicken fat (X₁), Triton X-100 (X₂), ammonium sulfate (X₃) and yeast extract (X₄).

Factor	Ss	df	Ms	F _{calc}	p-Value
X ₁ (L)	0.8438	1	0.84375	0,26904	0,612695
X ₁ (Q)	10.0233	1	10.02334	3.19601	0.097140
X₂ (L)	22.1184	1	22.11840	7.05261	0.019797
X ₂ (Q)	1.0209	1	1.02094	0.32553	0.578030
X₃ (L)	15.2642	1	15.26415	4.86708	0.045975
X ₃ (Q)	5.5296	1	5.52960	1.76315	0.207076
X₄ (L)	33.6540	1	33.65402	10.73082	0.006022
X ₄ (Q)	0.0074	1	0.00735	0.00234	0.962125
X ₁ x X ₂	12.3904	1	12.39040	3.95077	0.068333
X ₁ x X ₃	6.9696	1	6.96960	2.22231	0.159890
X ₁ x X ₄	0.0196	1	0.01960	0.00625	0.938193
X ₂ x X ₃	13.2860	1	13.28603	4.23634	0.060195
X ₂ x X ₄	0.4556	1	0.45562	0.14528	0.709245
X ₃ x X ₄	0.0870	1	0.08703	0.02775	0.870264
Error	40.7706	13	3.13620	-	-
Total SS	159.6361	27	-	-	-

Ss: Sum of squares; df: degrees of freedom; Ms: mean square; R² = 0.738; R = 0.859; F_{tab} (1;13; 0.05) = 4.67.

experiment, increasing the concentrations of chicken fat is not important for increasing the extracellular lipolytic activity.

On the other hand, the runs varying the concentration of Triton X-100 showed a significant effect for increasing the extracellular lipolytic activity, though at a reduced biomass (runs 19, 20 and 25). Rapp (1995) reported inducing effect of another surfactant, Span 85, in the formation of extracellular lipase from *F. oxysporum* sp. *vasinfectum*. Messias et al. (2009), Silva et al. (2005) and Mahadik et al. (2002) also reported increased production of lipase with different concentrations of Triton X-100 from *Botriosphaeria ribis*, *Metarhizium anisopliae* and *Aspergillus niger*, respectively. The mechanism for this effect, however, is not yet understood. The increased activity in cultures of *Fusarium* sp. (GFC) FCLA-MA41 with Triton X-100, reported in this study, is not due to the influence of the surfactant on the structure of the lipase. This statement is based on experimental observation after incubation of the lipase from *Fusarium* sp. (GFC) at different concentrations of Triton X-100 (0.0016 to 0.80 mM). It was observed that increase in activity of max 3% for concentrations below 0.22 mM, critical micelle concentration (CMC) for Triton X-100. Above the CMC, there was inhibition of activity at 0.5, 3.5, 9.5 and 13.5% for surfactant concentration of 0.32, 0.48, 0.64 and 0.80 mM, respectively.

Yeast extract is a complex nutrient, with peptides, amino acids and vitamins, being expected and its addition to the culture medium would provide more biomass. It is interesting to note, however, that the increase in the con-

centration of yeast extract favored the increase of extracellular lipase activity, but had no effect on biomass (runs 9, 10, 23 and 24). Ammonium sulfate, instead, favored growth but had no effect on enzyme production (runs 14 and 15). Rapp (1995) found the necessity of adding peptone for formation of extracellular lipase by *F. oxysporum* sp. *vasinfectum*. To biomass production there was no interaction between the variables at a confidence level of p < 0.05. However, when considered in isolation, chicken fat (X₁) and ammonium sulfate (X₃) showed a positive influence on the biomass production, and therefore increasing these variables led to an increase in biomass production of 5.47 and 2.40 g/L, respectively.

The production of enzyme has been associated, in the literature, with cell growth (Ghosh et al., 1996; Zarevúcka et al., 2005). However, runs 6 and 22 (Table 1), for example, show opposite relation of Lipase Activity/Biomass (767 and 27.6, respectively). As the factorial design was carried out at a fixed time of 72 h, to confirm the difference in activity was due to experimental condition and not because of the time of cultivation, runs 6 and 22 were repeated with analysis every 24 h (Figure 2). In both cases, the maximum activity was found in the stationary phase of cell growth. However, the results confirmed that despite higher biomass in condition 22, the extracellular lipolytic activity is lower compared to run 6 in the condition in which growth is reduced at higher lipolytic activity. It was found that the lipase production from *Fusarium* sp. (GFC) FCLA-MA41 is not directly related to the increased number of cells, but due to the metabolic effect or transport (Table 2). Rapp (1995) found that

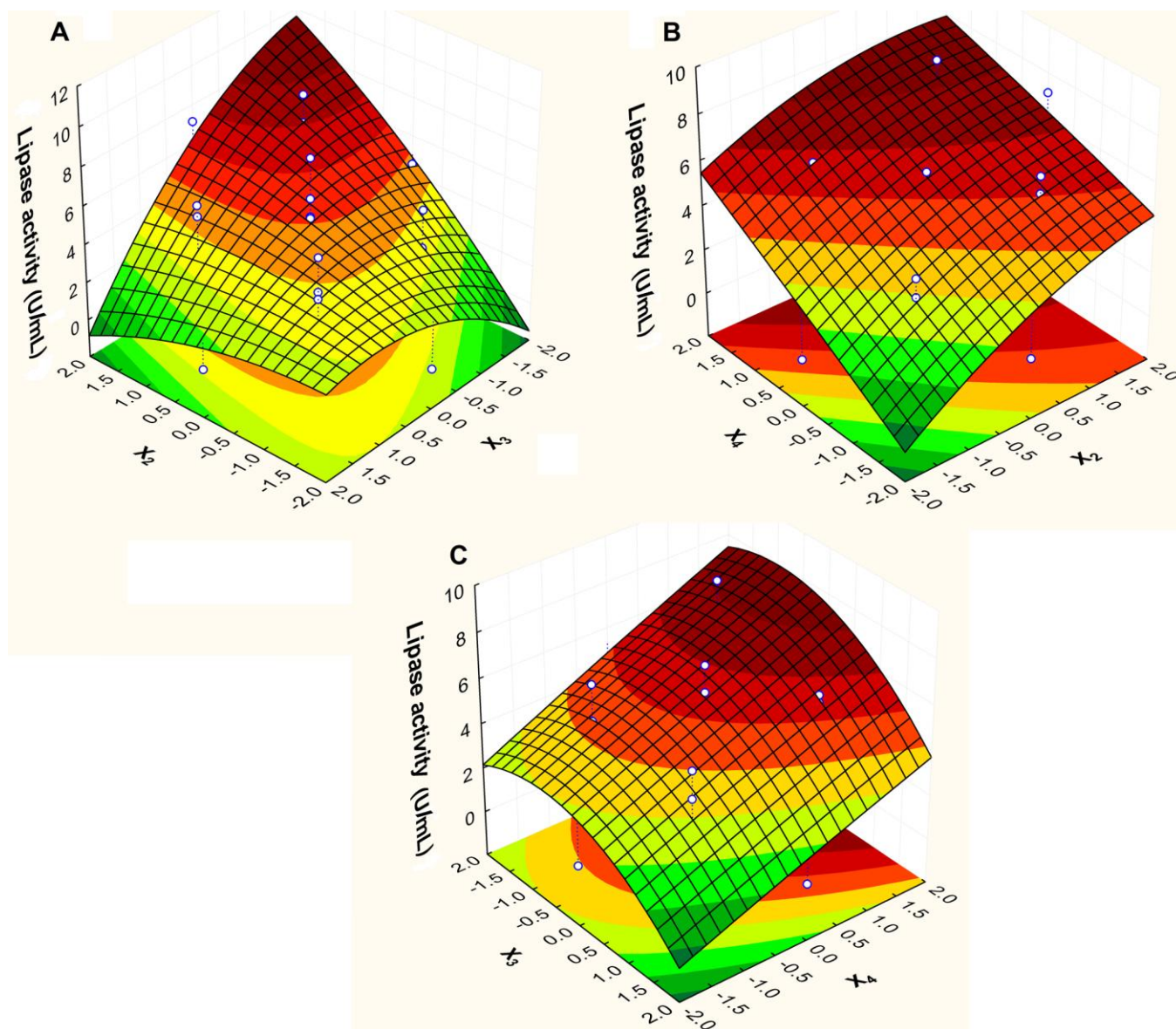


Figure 1. Three-dimensional response surface of lipase activity (U/mL) obtained by submerged fermentation of *Fusarium* sp. (GFC) FCLA-MA-41 in function of X_2 and X_3 (A), X_2 and X_4 (B) and X_3 and X_4 (C), on medium with various concentrations of chicken fat (X_1), Triton X-100 (X_2); ammonium sulfate (X_3) and yeast extract (X_4).

during growth of *F. oxysporum* f. sp. *vasinfectum* in shake flasks, a small amount of lipase activity was found to be associated with the mycelium.

The authors suggested that lipase activity is associated for some time with the cell wall in the course of its secretion into the culture medium. The assumption was made for the secretion of lipase activity from *Geotrichum candidum* (Tsujiisaka et al., 1973), *Serratia marcescens* (Winkler and Stuckmann, 1979) and *F. oxysporum* f. sp. *lini* SUF.8 (Hoshino et al., 1991). Therefore, the increase in activity with the addition of Triton X-100, with no

increase in biomass, may be due to the detergent effect by increasing the permeability of the cell wall, and consequently increasing the secretion of the enzyme by the cell.

Although, the cost of culture medium is an important parameter to determine the economic viability of a bio-process, the upstream process are more expensive and may represent up to 80% of the total costs of enzyme production (Keller et al., 2001). Thus, the priorities are nutrients that contribute to increased production but preferably do not result in difficulties in recovering, purifying

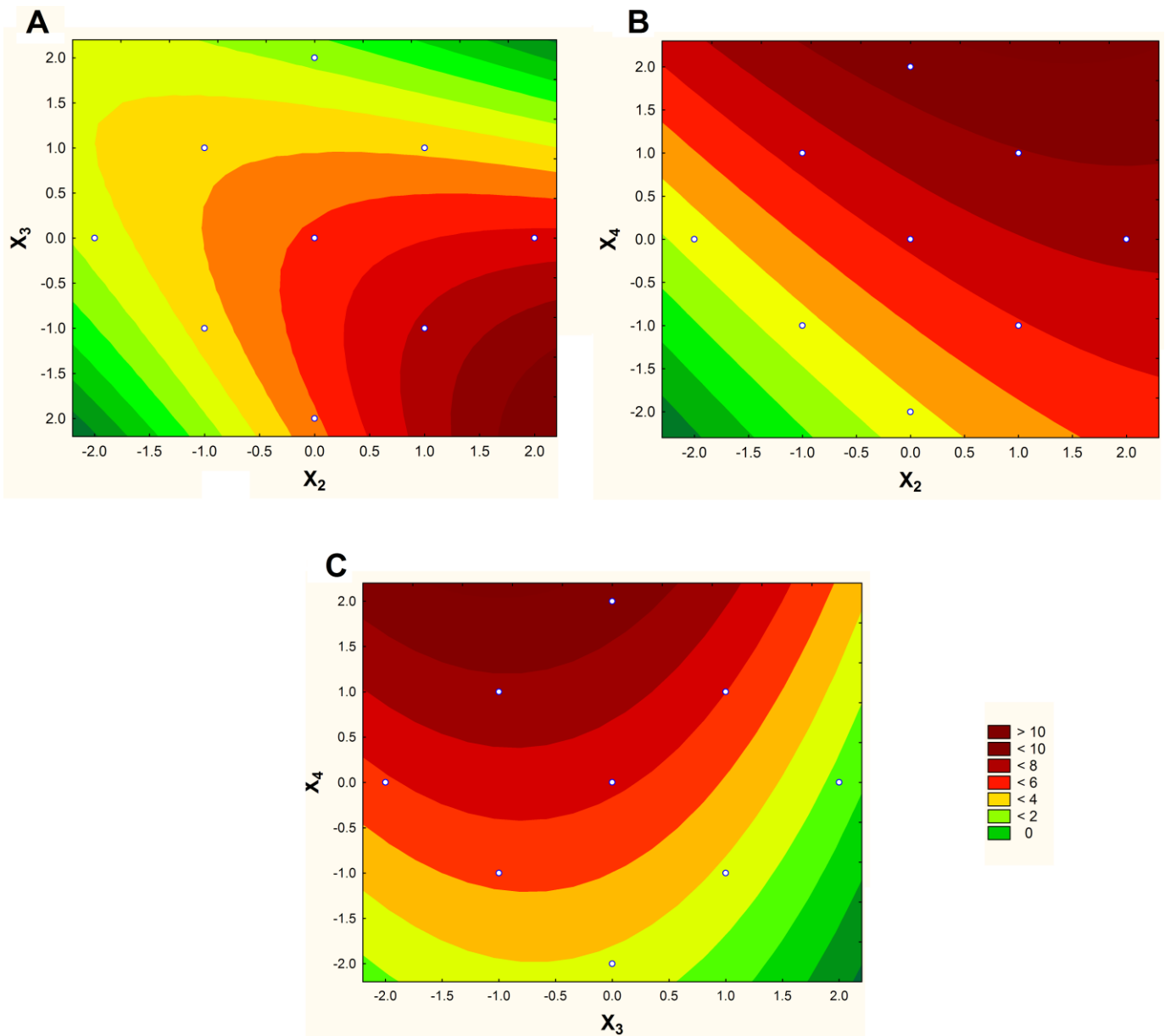


Figure S1: Contour plots of lipase activity (U/mL) obtained by submerged fermentation of *Fusarium* sp. (GFC) FCLA-MA-41 in function of X_2 and X_3 (A), X_2 and X_4 (B) and X_3 and X_4 (C), on medium with various concentrations of chicken fat (X_1), Triton X-100 (X_2); ammonium sulfate (X_3) and yeast extract (X_4).

and applying the enzyme by increasing the number of upstream process. In the present study, VSMS represents 33.46% of the production cost and it is essential for the production of lipase (activity is 98% lower in its absence – data not show), is comprised only of inorganic salts whose residues are easily separated at the end of fermentation. Yeast extract, in contrast, are very expensive and have a complex composition including proteins and peptides, which complicate the purification. The results are important because they demonstrate the possi-

bility of combining organic and inorganic sources for the production of lipase from *Fusarium* sp. (GFC) FCLA-MA41 reducing the concentration of yeast extract. The production was optimized through the use of ammonium sulfate, this way; a cheaper ecologic culture medium for lipase production is feasible.

Previous study with the same *Fusarium* sp. (GFC) FCLA-MA41 strain in SmF with culture medium containing crambe oil (17.5 mL/L), Triton X-100 (5 g/L), ammonium sulfate (5 g/L) and yeast extract (1 g/L) was

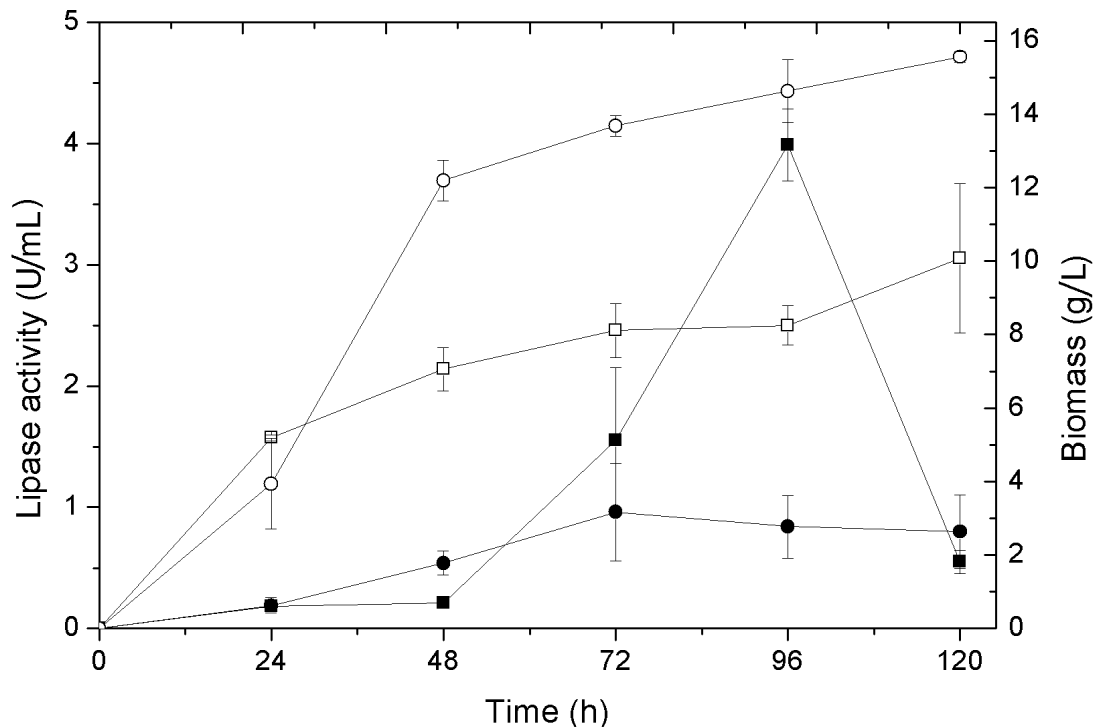


Figure 2. Curve of growth and extracellular lipase activity in cultures of *Fusarium* sp. (GFC) FCLA MA-41. Run 6: (■) Lipase activity, (□) Biomass. Chicken fat 15.0 mL/L, Triton X-100 15.0 g/L, ammonium sulfate 4.5 g/L and yeast extract 0.75 g/L. Run 22: (●) Lipase activity, (○) Biomass. Chicken fat 20.0 mL/L; Triton X-100 10.0 g/L, ammonium sulfate 19.0 g/L and yeast extract 0.5 g/L.

proposed, resulting in a lipase titer of 3.0 ± 0.25 U/mL (Oliveira et al., 2013). Although providethis provides lower activity than that obtained with chicken oil, the cost per unit of enzyme activity was also lower (\$US 183.00 and \$US 518.00/million Units of lipase with crambe oil and chicken fat, respectively). Still, the economic viability of the bioprocess is also related to other factors such as availability of raw materials. The crambe oil has the advantage of being a non-food oil seed, however, currently is cultivated as an oil-seed on a large scale in Mexico, New Zealand, Russia, United States, but most crambe oil processing occurs in Europe (Warwick and Gugel, 2003) for industrial uses. The poultry processing, by contrast, has a worldwide distribution and the oil/fat is an inexpensive residue.

Conclusion

The findings of this work have demonstrated good results of lipase production from *Fusarium* sp. (GFC) FCLA-MA41 by SmF. The fungal isolate was found to be a producer of lipase with market potential, as lipase activity was obtained on nutrient medium containing low-cost nutrients, such as ammonium sulfate and chicken fat. The lipase activity obtained was 4.22 U/mL and equated

to a cost of \$US518.00/million units of lipase. Moreover, the use of chicken oil as an inducer for lipase production is unprecedented.

Conflict of Interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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Full Length Research Paper

Synthesis of liposomes using α -phosphotidycholine and metabolites obtained from *Elephantorrhiza elephantina* and *Pentanisia prunelloides*

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Plant saponins exhibit numerous pharmacological characteristics desirable for long term hyperlipidemic therapy through their cholesterol binding capacity due to the formation of liposomes/phytosomes which ultimately decreases the gastrointestinal absorption of cholesterol. This may result in the reduction of the blood plasma cholesterol levels; hence, mitigating cardiovascular and atherosclerotic problems that are associated with elevated plasma cholesterol concentrations. In this study, we reported for the first time a potential method of synthesizing phytosomes/liposomes from two medicinal plants *Pentanisia prunelloides* (Rubiaceae) and *Elephantorrhiza elephantina* (Fabaceae) saponin extracts and fractions using α -phosphotidycholine and cholesterol. This was done to explore the possibility of cholesterol binding capacity of fractions and extracts of the two medicinal plants as a justification of their application by traditional healers in managing body weight as well as averting hyperlipidemia and atherosclerosis. Spherical nanoparticles/phytosomes/liposomes were clearly identified in the TEM images with particle sizes ranging between tens and hundreds of nanometers. The zeta potential of the nanoparticles fell between -5 and +5 mV indicating that they have a high potential for aggregation; hence, making it relatively very difficult for the complexed cholesterol molecules to permeate the microscopic pores in the alimentary tract.

Key words: Frontier transmission infra-red (FTIR), liposomes, adjuvants, zeta-potential, α -phosphotidycholine.

INTRODUCTION

Recently, much phytochemical-research attention has focused on the metabolites and pharmacokinetics of saponins due to their biological activities and absorption profile (Akihisa et al., 2007; Donya et al., 2007; Harinantenaina et al., 2006; Kimura et al., 2005). Saponins are responsible for diverse effects including anti-inflammation, anti-allergy, antitumor, augmentation of the immune responses, stimulating the apoptosis of skin cells, anti-obesity and anti-hyperlipidemia (Awad et al., 2011; Amin et al., 2010; Wang et al., 2011). Saponins are

believed to form the main constituents of many drugs and folk-medicines and are considered responsible for numerous pharmacological properties (Liu and Henkel, 2002). Notably, saponins can activate the mammalian immune system, which has led to significant interest in their potential as vaccine adjuvants/liposomes (Skene and Sutton, 2006). Vaccines require optimal adjuvants including immunopotentiator and delivery systems to offer long term protection from infectious diseases in animals and man. Phospholipids like alpha- phosphodylcholine

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are thus employed in the formation of phytosomes/ adjuvants as natural digestive aids and as carriers for both fat-miscible and water miscible nutrients. Saponins on the other hand are generally used as adjuvants/ liposomes to enhance bioavailability of medication.

Adjuvants/liposomes influence the titer, duration, isotype and avidity of antibody, and affect the properties of cell-mediated immunity (Hunter et al., 1995; Kaeberle, 1986; da Silva et al., 2005). The common clinically used adjuvants are synthetic and are mostly synthesized with the inclusion of aluminium. These adjuvants have disadvantages like side effects, strong local stimulation and carcinogenesis accompanied by complicated preparations (Bowersock and Martin, 1999). This therefore calls for the preparation of physiologically friendly phytosomes; hence, this study on the use of secondary metabolites from *Elephantorrhiza elephantina* and *Pentanisia prunelloides* as potential adjuvant/phytosome precursors. The lead candidate saponin adjuvants or liposomes are Quillaja A and its derivatives Quillaja Saponin-21 (Kensil and Kammer, 1998). Quillaja saponins have been reported to have a high degree of toxicity and hence, confer undesirable haemolytic effects apart from their instability in aqueous phases which would limit their application as phytosomes/liposomes in human vaccination (Cox et al., 1999; Waite et al., 2001; Marciani et al., 2003). On the other hand, the overexploitation of the soap bark tree from which Quillaja-A saponin is obtained, *Quillaja saponaria* has caused important ecological damage and considerable shortage of available supplies (Marciani et al., 2003).

Triterpenoid glycosides were found to be predominant in both *E. elephantina* and *P. prunelloides*; hence, allaying the haemolytic effects due to the administration of the two plant metabolites in phytotherapy. Hence, the exploitation of saponins from *E. elephantina* and *P. prunelloides* could act as an alternative to Quillaja saponins. Due to the problematic nature of isolating pure saponins from plants, many reports assessing the hypocholesterolemic activity of plant saponins use 'saponin fractions containing multiple related structures rather than individually isolated saponins (Hazai et al., 1992; Southon et al., 1988; Malinow et al., 1987a,b; Oakenfull, 1986). It is for this reason that crude extracts and partially isolated saponins from *E. elephantina* and *P. prunelloides* were used in this study.

MATERIALS AND METHODS

Plant material and collection

Fresh plant rhizomes of *E. elephantina* and *P. prunelloides* were collected in June 2010 from seven different Southern African regions in Swaziland, South Africa and Zimbabwe and identified by Dr Anna Moteetee, University of Johannesburg. Voucher specimens (SJM-0, SJM-01 to SJM-08) were deposited in JRAU Herbarium, Department of Botany and Plant Biotechnology (Kingsway Campus, University of Johannesburg). Fresh plant rhizomes were washed with water, marcerated, air-dried at ambient temperature and kept in the fumehood at room temperature. The dried plant ma-

terials were then ground into fine powders, extracted as described as follows and solvents evaporated under reduced pressure while aqueous extracts were dried using the freeze drier. The dried samples were then stored in sample bottles at room temperature.

Qualitative determination of secondary metabolites in *E. elephantina* and *P. prunelloides*

The dried ground rhizome powders with a moisture content of about 10% were extracted with n-hexane three times until the solvent became clear. The extract was filtered and concentrated *in vacuo* at 40°C. The same procedure was applied consecutively with chloroform, ethyl acetate, methanol and water and percentage yields were then calculated. Standard qualitative tests were carried out (Edeoga et al., 2005; Harbone et al., 1973; Speedie and Tyler, 1996) on the crude extracts and fractions of *E. elephantina* and *P. prunelloides* as well as the Liebermann-Burchard's test for trieterpenes and sterols (Cook, 1961).

Quantitative determination of saponins

A mass of 20 g powdered sample was mixed with 100 ml of 20% aqueous ethanol. The mixture was heated in a water bath for 4 h at 55°C with constant stirring then filtered. The residue was re-extracted further with another 100 ml 20% ethanol. The combined extracts were reduced to 40 ml over a water bath at 90°C. The concentrate was transferred to a 250 ml separatory funnel partitioned with 3 × 20 ml diethyl ether shaking vigorously. The aqueous layer was recovered while the ether layer was discarded. Partitioning of the solution was done three times with 60 ml portions of n-butanol. The combined n-butanol fractions were washed twice with 10 ml of 5% aqueous sodium chloride. The resultant solution was heated in a water bath for the vaporization of n-butanol. The final dried fraction was further dried to constant mass in an oven to constant mass. The saponin content was calculated as a percentage of the starting dried plant material.

Thin layer chromatography (TLC) of extracts

Aluminum-backed 0.3 mm silica gel 60 F₂₅₄ pre-coated tlc plates (cut into 5 × 10 or 10 × 10 cm) were used. Each crude extract (0.1 µL) was placed on the TLC plate 0.30 mm silica gel 60 with fluorescence indicator UV₂₅₄ Alugram Sil G UV₂₅₄ type. Portions of both *E. elephantina* and *P. prunelloides* were spotted on separate TLC plates and eluted with butanol/acetone/water mixture (4:1:2). The developed tlc plates were sprayed with vanillin sulphuric acid and then heated in the oven at 110°C for 5 min. Portions of both plant extracts were subjected to column chromatography using the same eluent applied for preparative PTLC. The resultant fractions of the two plant extracts were spotted on the same TLC plate against standard digitonin and saponin from Quillaja (Figure 3). This was done for comparison purposes. Preparative TLC was then used to isolate the major compounds with TLC3 (MeOH/H₂O/acetone/ethyl acetate/chloroform) 10:8:30:40:12 as eluent. Standard digitonin and Quillaja-21 were also spotted against the isolates for comparison purposes.

Column chromatography analysis of extracts

The dried residue (10.0 g) was suspended in minimum amount of methanol, immobilised on silica gel (8 g), and subjected to column chromatography (CC), using a 38 × 4.5 cm glass column filled with Merck silica gel 60 F₂₅₄ in chloroform to a level of about 8 cm from the top. The immobilized extract was added to the free volume at the head of the column. Fractionation was done by successive

Table 1. Preparation of nanoparticles/phytosomes.

Origin of saponin	Code	Saponin mass (g)	Cholesterol mass (g)	Volume of phospholipid (ml)	Volume of methanol (ml)
Zim Ee	SJ 1	0.0141	0.0138	1.5	10
KZN Ee	SJ 2	0.0154	0.0253	1.6	10
O. F. Pp	SJ 3	0.0141	0.0198	1.4	10
Sicunusa Pp	SJ 4	0.0135	0.0145	1.3	10
Saponin from Quillaja	SJ 5	0.0143	0.0143	1.2	10

Table 2. Percentage yields of moisture-free dried powders of *P. prunelloides* and *E. elephantina* for different solvents.

Extraction solvent	Sample origin and percentage yields (%)			
	Zim. (Ee)	KZN (Ee)	O. Farm (Pp)	KZN (Pp)
n-hexane	0.059	0.039	0.093	0.62
Chloroform	0.12	0.15	0.41	0.25
Ethyl acetate	0.12	0.14	0.09	0.15
Methanol	19.6	22.68	17.12	7.2

applications of 600 ml of each of the following solvents; hexane, chloroform, methanol and water. Four major fractions were collected and the solvent was evaporated in the fume hood overnight and the water extracts were dried by means of a freeze drier. The resultant fractions were weighed and percentage yields evaluated as depicted on Table 2.

FT-IR spectroscopic analysis extracts

A 2 mg of powdered plant sample was mixed with 300 mg of spectroscopic grade purity and spectroscopically dry Kbr salt in an agate mortar using a pestle, and compressed into thin pellets. Infrared spectra were recorded between 4000 to 500 cm^{-1} as Kbr pellets on the TL 8000 balanced flow FT-IR EGA (Perkin Elmer Spectrum series) instrument.

Preparation of nanoparticles using α -phosphatidylcholine as precursor

Phytosomes are prepared in different ways depending on the precursor secondary metabolites and phospholipids used (Karate et al., 2013). The preparation of nanoparticles in this study was carried out as summarized on Table 1 which is similar to literature reports (Yanyu et al., 2006; Maiti et al., 2006). *E. elephantina* (Ee) samples obtained from Zimbabwe (Zim-SJ 1) and Kwazulu Natal (KZN-SJ 2) while *P. prunelloides* (Pp) samples were from Orange Farm (O.F.-SJ 3) and Sicunusa-SJ 4 and the standard saponin from Quillaja-SJ 5 were mixed with α -phosphatidylcholine, cholesterol and methanol in the respective proportions summarised in Table 1. All the chemicals (Methanol, Quillaja saponin, cholesterol and L- α -phosphatidylcholine) used in this study were of high purity purchased Merck.

Characterization of the synthesized nanoparticles

Nanoparticle size and zeta potential

To determine the size of the particles, 5 ml sample was sonicated after re-dispersing in methanol and placed into the analyzer

chamber. Readings were collected at 25°C with a detector angle of 90° using a Malvern Zetasizer and Particle Analyzer 5000.00 (Malvern Instruments UK) at the University of Johannesburg.

Morphology study

The morphology of the nanoparticles was studied by a TEM using a JEOL Electron Microscope JEM 2100 (2000.00 kV) at CISR in Pretoria. The samples were dispersed in methanol, sonicated for 10 min and mounted on carbon coated copper grids before examination. The size, shape, membrane integrity, aggregation and fusion between vesicles were examined by means of TEM over a week during 21 days. Results for the particle sizes are shown on Table 5 (Reis et al., 2006) and for the TEM images (Figures 5 to 7).

RESULTS AND DISCUSSION

Qualitative phytochemical profiles

Phytochemical tests were carried out on the crude extracts (Table 3) as well as different fractions from the sequential fractionation (Mpofu, 2014; University of Johannesburg, PhD thesis submitted in 2013). The procedure was undertaken to confirm the partitioning of various secondary metabolites in the respective solvents applied with saponins and their derivatives expected to be predominantly found in the methanolic and aqueous fractions. The appearance of an intense reddish colour in the Liebermann-Burchard's reagent for both aqueous and methanol fractions was indicative of the presence of tri-terpene saponins. UV-Vis spectra for the saponin fractions from the two plant extracts were indicative of the presence of the respective secondary metabolites which were conspicuous by their poor chromophores (Figure 1). The UV-Vis spectra for all the saponin fractions as

Table 3. The phytochemical profile of crude extracts of *E. elephantina* and *P. prunelloides* from different locations.

Major phytochemical	Sample origin and estimation of major phytochemicals			
	Zim. (Ee)	KZN (Ee)	O. Farm (Pp)	KZN (Pp)
Alkaloids	+	+	+	+
Saponins	+++	+++	++	++
Anthraquinones	+++	+++	++	++
Tannins	+++	+++	++	++
Flavonoids	+++	+++	++	++
Anthocyanidins	+++	+++	++	++

+ = Trace amount, ++ = detectable, +++ = substantially detectable, - not detectable.

Table 4. The relative saponin percentage composition of *E. elephantina* and *P. Prunelloides*.

Sample	Origin of sample	Saponin percentage (%)
<i>E. elephantina</i>	Zimbabwe	1.56
	KZN	1.44
<i>P. prunelloides</i>	Orange farm	0.24
	Sicunusa	0.71

Table 5. Zeta potential values of nanoparticles synthesized from *E. elephantina* and *P. prunelloides*.

Sample	Z-potential (mV)
SJ1 (fresh)	-4.65
SJ2 (fresh)	+0.829
SJ1 (after 3 weeks)	+2.15
SJ2 (after 3 weeks)	+0.22
SJ3	-1.38
SJ4	-0.853
SJ5	+0.807

reflected in Figure 1 exhibited a very poor chromophore with λ_{\max} in the range of 208 to 210 nm which is in agreement with literature. The UV-Vis spectra for all twelve fractions of saponins of *E. eepphantina* and *P. prunelloides* drawn from four different geographical locations were superimposed (Figure 1) which suggested selectivity of the extraction technique applied.

Rhizomes of both plants exhibited all the six different secondary metabolites tested although in varying degrees. Saponins and flavonoids tended to be more pronounced for both crude extracts and fractions as depicted by the intensity of colours across all samples from different geographical locations (Table 3). A further analysis showed *E. elephantina* to have a relatively greater content of the two classes of phytochemicals as exhibited by the intensity of the colour changes for the prescribed standard tests. These two classes of compounds are generally polar and this observation tentatively corroborated

the findings on the quantitative determination of fractions eluted by solvents of increasing polarity (Table 2) that exhibited more polar compounds from the two species. The qualitative tests demonstrated the abundance of the major secondary metabolites that could lend support to the extensive use of the two medicinal plants in phytotherapy. Despite the fact that the samples were drawn from different geographical locations, the difference in the saponin content between these two medicinal plants was consistently conspicuous and confirmed the observations in the qualitative tests.

Quantitative composition of saponins in *E. elephantina* and *P. prunelloides*

The equantification of saponins was adopted from (Ediogo, et al., 2005 and Astuti, et al., 2011). The relative percentage composition of saponins in the two plants is shown in Table 4. *E. elephantina* samples exhibited a higher quantity of saponins (1.56 and 1.44%) compared to *P. prunelloides* samples (0.24 and 0.71%) (Table 4). Despite the fact that the samples were drawn from different geographical locations, the difference in the saponin content between the two species was conspicuous. The intensity of the precipitate formed was more pronounced for both *E. elephantina* extracts compared to both aqueous and methanol *P. prunelloides* extracts. This again suggested a relatively higher saponin content of this class of phytochemicals in *E. elephantina*. This group of phytochemicals is very important in phytotherapy and may have a significant role in the treatment of ailments for which these two medicinal plants are pre-

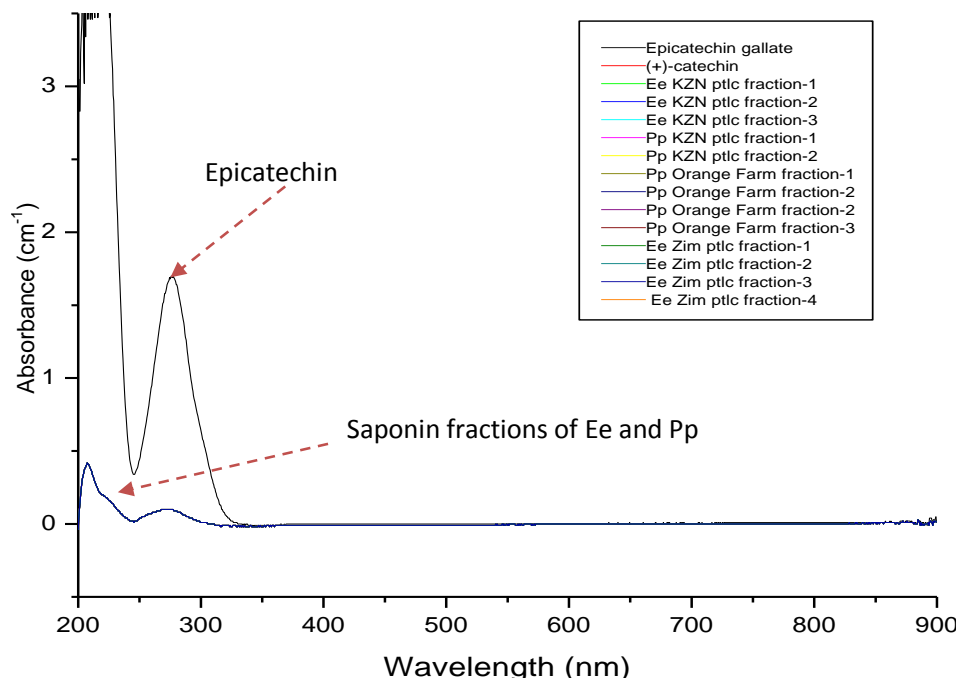


Figure 1. The UV-Vis spectra of *E. elephantina* (Ee) and *P. prunelloides* (Pp) fractions from different geographical locations (Kwazulu Natal, KZN; Zimbabwe, Zim; Orange Farm, OF).

scribed by traditional healers. Furthermore, both groups of secondary metabolites are good candidates for the formation of phytosomes which augers well for the application of these two plants in the phytopharmaceutical industry. The health benefits of saponins include the reduction of cholesterol levels in the intestinal tract; hence, mitigating obesity and antimutagenicity thus preventing cancer cells from growing. The non sugar parts of saponins have a direct antioxidant activity; hence, reducing the risk of cardiovascular disorders. It is most likely that the antioxidant capacities exhibited by extracts of these two medicinal plants emanates partly from this group of phytochemicals apart from the flavonoids and other secondary metabolites yet to be discovered. The relatively high content of saponins suggests that both *E. elephantina* and *P. prunelloides* have cytotoxic effects such as intestinal cell membrane permeabilization.

Saponins are reported to mimic the sex hormone involved in controlling the onset of labour in women and the subsequent release of milk (Okwu and Okwu, 2004). It is most likely that the efficacy of concoctions of *P. prunelloides* administered to expectorant women and those that deliver without the release of the placenta for the enhancement of ease delivery (Okwu, 2003) emanates partly from this class of compounds.

TLC for the fractions of *E. elephantina* and *P. prunelloides* against saponin standards

The green colour after spraying with vanillin sulphuric acid and baking confirmed the presence of saponins in

the methanol extracts of *E. elephantina* (Ee) and *P. prunelloides* (Pp) (Figure 2). Furthermore, TLC analysis of saponin fraction against digitonin afforded sub-fraction 61 of *E. elephantina* which exhibited the presence of saponins and triterpenoids as depicted by the green and purple colours, respectively. The preparatory TLC for fraction 59 afforded five sub-fractions that exhibited the purple colour for triterpenoid after spraying with vanillin sulphuric acid and baking. Both fractions 61 and 59 of *E. elephantina* gave positive results from Liebermann-Burchard and Molish reactions which suggested that they were trietepenoid saponins.

Frontier transmission infra-red (FT-IR) spectra of saponins from *E. elephantina*

Some functional groups in the saponin fractions of *E. elephantina* and *P. prunelloides* were examined using FTIR spectrometry and the results are shown in Figures 3 and 4. A peak at 3459.16 cm^{-1} was displayed from the spectrum indicative of the presence of hydroxyl group. The peaks ranging from 3016.75 to 2925.56 cm^{-1} suggested the presence of C-H while the intense peak at 1738.93 to 1739.00 cm^{-1} were indicative of the presence of carbonyl groups (C=O) and asymmetric C=O stretching in the carbohydrates were also suggested by the peak at 1435.19 cm^{-1} (Yim et al., 2007). Oligosaccharide linkage to saponins absorption bands were evidenced by the C-O-C bands, between 1052.75 and 1092.63 cm^{-1} (Zheng et al., 2008). The peak at 1235.54 cm^{-1} may be indicative of C-O stretching in ether or alcohols. The peaks 904 and 727

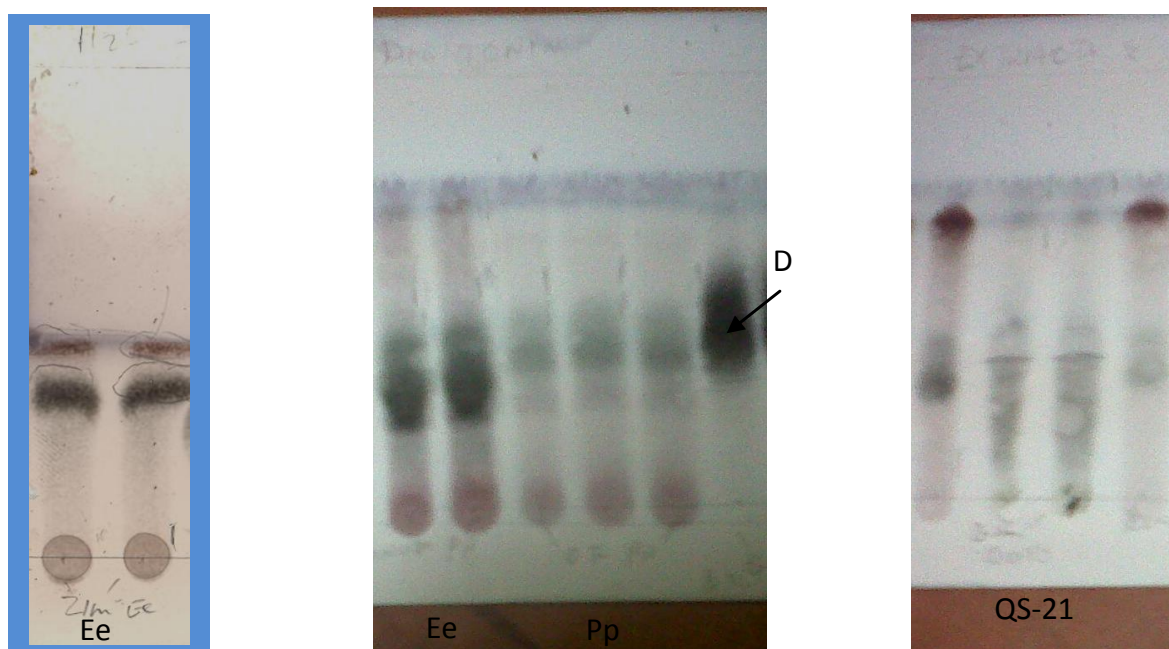


Figure 2. TLC for *E. elephantina* (*Ee*) and *P. prunelloides* (*Pp*) saponin fractions after baking at 110°C against digitonin (D) and quillaja saponin (QS-21) standards.

were assigned as characteristic absorption bands of carbohydrates and their derivatives according to Nakanishi and Solomon (1977) and Deng et al. (2003). The stronger the relative intensity of the absorption bands between 1150 and 700 cm^{-1} , the higher the starch content (Nakanishi et al., 1977; Li et al., 2004; Hua et al., 2003; Woo et al., 1999). The difference in sugar chain of fraction 59-2 and 59-4 could therefore be explained by the fact that the former fraction eluted earlier than the latter due to steric reasons. The $-\text{OH}$, $\text{C}-\text{H}$, $\text{C}=\text{C}$, $\text{C}=\text{O}$ and $\text{C}-\text{O}-\text{C}$ absorption bands found in *P. prunelloides* and *E. elephantina* are suggestive of Oleonane-type triterpenoid saponins as confirmed by the Liebermann-Burchard and Molish reactions as well as liquid-chromatography-electrospray-ionisation-mass spectrometry (LC-ESI-MS). These oleonane-type triterpenoid saponins are characterized by the $\text{C}=\text{O}$ infrared absorbance due to acid/ester linkages. Such triterpenoid saponins are also likely to be bidesmosides since they have two attachments for glycones (that is, glycosidic and ester groups) to the sapogenin.

The aforementioned referred infrared functional group absorptions characteristic of saponins are also cited in these literature (Kirmizigul and Anil, 2002; Natori et al., 1981; Da Silver Bernadete et al., 2002).

Particle size and zeta potential for *E. elephantina* and *P. prunelloides* nanoparticles

Zeta potential is the measure of the magnitude of the repulsive or attractive electrical forces that exists between atoms, molecules, particles and cells in a fluid. Its

measurement relates to the overall charge of particles to the same extent but it also relates to the stability of particles in dispersion. An increase in the 'zeta potential' in solution allows it to dissolve and hold more material. When 'zeta potential' is too low, blood begins to coagulate making it difficult for the heart to pump it through the circulatory system. This is a condition known as intravascular coagulation which may lead to many cardiovascular problems. There are numerous causes of cardiovascular problems for example, processed foods which have a tendency of reversing nature's ratios of K^+ to Na^+ , artificial farming methods applying cationic herbicides and pesticides that have a tendency of reversing the zeta potential, increased use of pharmaceutical drugs, over 90% of which are strongly cationic and the use of strongly cationic cans of food and drinks (Riddick, 1968). These different causes of cardiovascular problems result in the ionic content of blood being skewed towards cationic species; hence, lowering the blood 'zeta potential' ultimately creating cardiovascular stress that causes intravascular coagulation. The higher the 'zeta potential' for example (-100 to -60 mV), the greater the stability and the lower the 'zeta potential' for example (-5 to +5 mV), the lower the stability of solution (Riddick, 1968).

A further analysis of the causes of cardiovascular diseases shows that they are associated with developed worlds in which most food materials are processed. The 'zeta potential' (mV) for the nanoparticles/phytosomes produced in this study are summarized in Table 5. Generally, all 'zeta potential' values for the phytosomes formed fell between -5 and +5 mV indicating that they are relatively unstable; and hence, have a high potential for

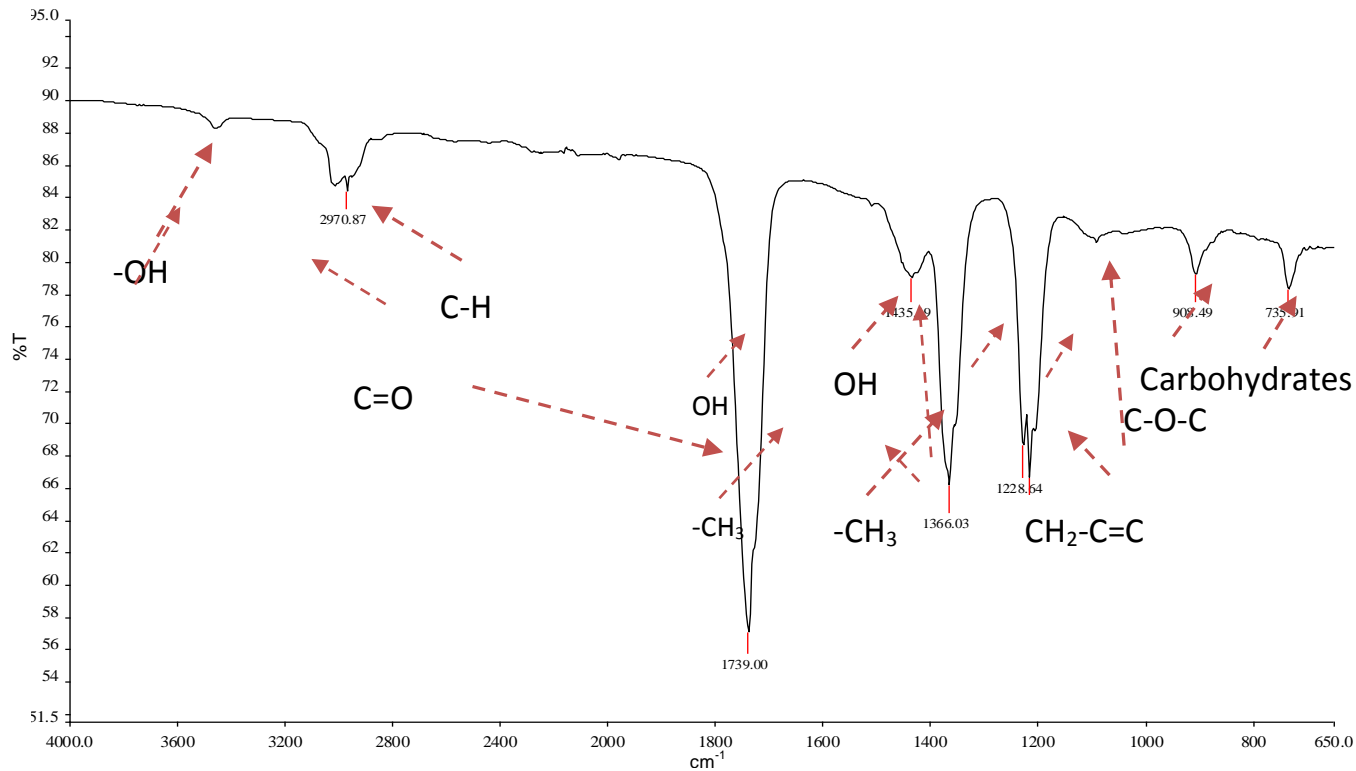


Figure 3. FT-IR spectra of *E. elephantina* isolate 59-2.

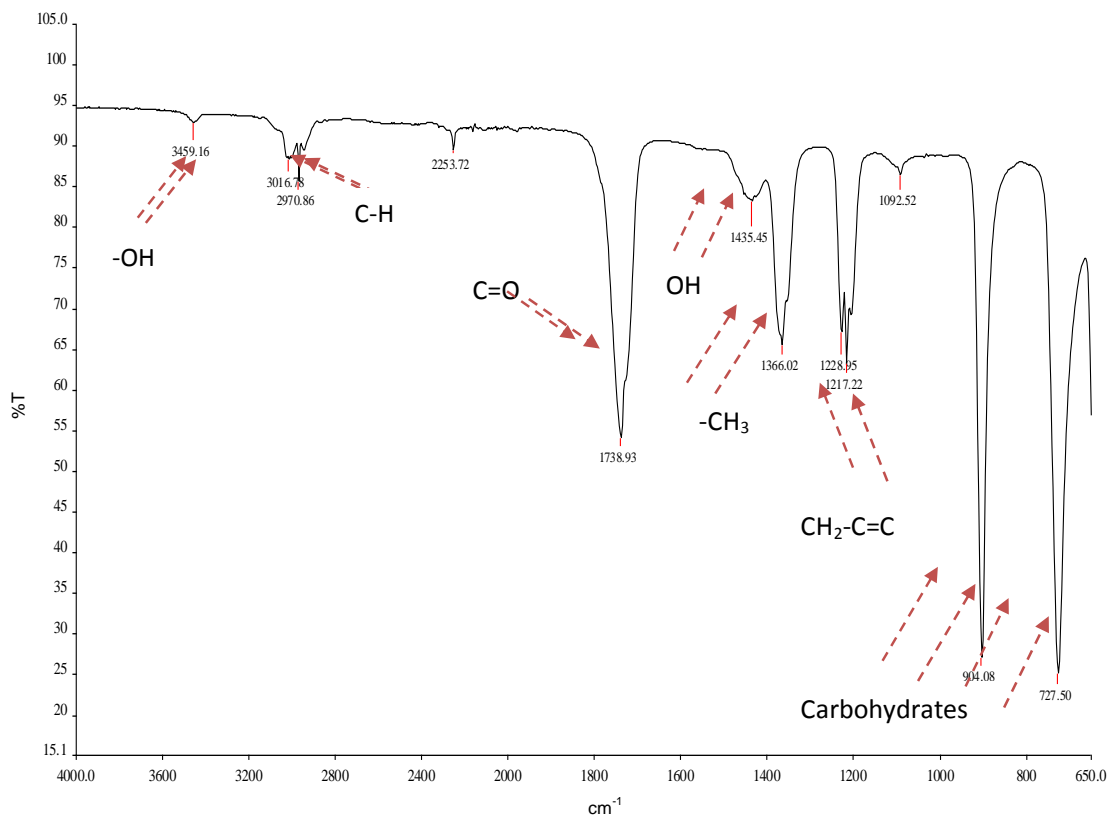


Figure 4. FT-IR spectra of *E. elephantina* isolate 59.4.

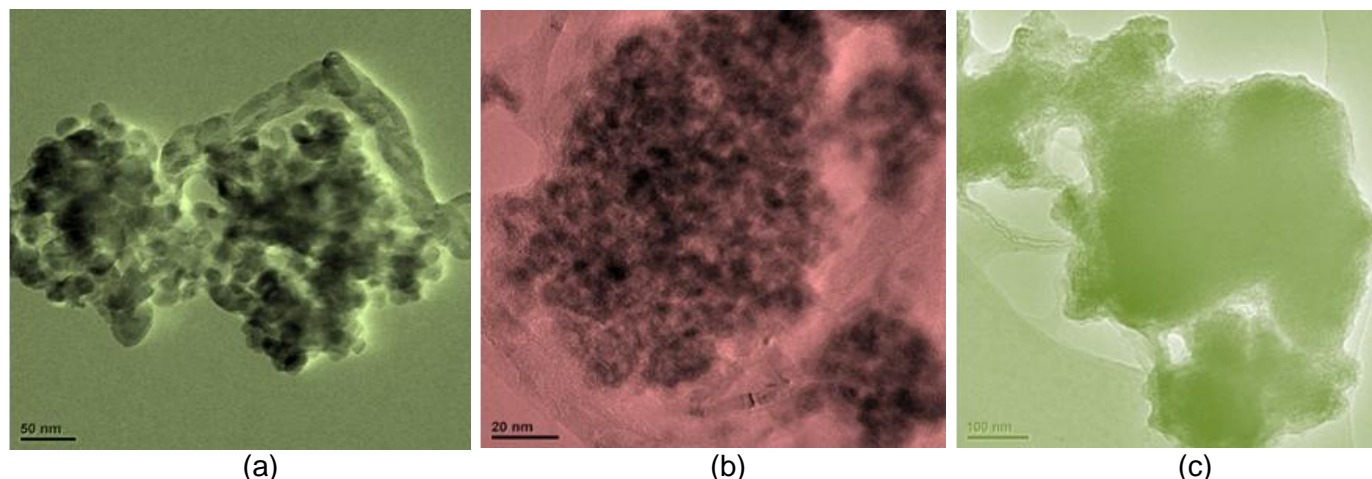


Figure 5. TEM: (a) Ee + cholesterol, (b) Pp + cholesterol, (c) cholesterol alone.

aggregation over time.

Morphology of nanoparticles

The particle size (nm) for samples of the phytosomes produced in this study are shown in Figures 5 to 7. The essence of L- α -phosphatidylcholine in the formation of phytosomes was clearly demonstrated by the TEM images in Figure 5a to 5b. In both cases, only cholesterol was added to either *E. Elephantorrhiza* (Ee) or *P. prunelloides* (Pp) resulting in an insignificant formation of distinct phytosomes. A close observation on Figure 5 suggests that the Pp fraction exhibits a greater potential to form phytosomes in the nano scale (20 nm) relative to Ee fractions (50 nm). Spherical monolayer nanoparticles/ phytosomes were clearly observed in the TEM images soon after the addition of L- α -phosphatidylcholine to the mixture (Figure 6). The size of the nanoparticles varied between tens to hundreds of nanometers in diameter that is, 50, 500 and 100 nm (Figure 6a, 6b and 6c, respectively). It should be noted that the TEM image (Figure 6b) for which nanoparticle size was 500 nm represented an aggregate of particles implying that the discrete particles had a far smaller size than 500 nm. This difference in the size of particles could be due to the difference in the nature of the saponins or secondary metabolites since they were derived from two different plants that is, 6a from Ee and 6b from Pp. Long sonication periods combined with nanofiltration in a microextruder system enabled to control the vesicle size and to produce relatively uniform vesicles (Figure 6a and 6c in particular). In order to evaluate the vesicle's stability, the saponin nanoparticles were left at room (temperature).

After a period of two weeks, some fusion and aggregation of vesicles appeared, indicating a relatively strong aggregation and precipitation capacity of the nanoparticles as depicted in Figure 7. The particle size rose from

the nano scale to the micro scale that is, from about 50 nm to 0.5 μ m (Figure 7a to 7c). This clearly demonstrated a high degree of aggregation of the phytosomes formed from these two medicinal plants. Oleanolic acid is one of the most common aglycones which has been reported to possess anti-viral, (anti-HIV), anti-inflammatory, hepato-protective, anti-ulcer, anti-bacterial, hypoglycaemic, anti-fertility and anti-carcinogenic activity (Liu, 1995, 2005). *E. elephantina* and *P. prunelloides* are used to remedy some of the aforementioned referred anomalies and the presence of the oleanolic acid aglycone may account for the pharmacological benefits of these two medicinal plants. The agglomeration of the nanoparticles of *E. elephantina* and *P. prunelloides* saponins as they were mixed with α -phosphatidylcholine may justify the use of the two plants for the reduction of body weight as it is anticipated that they accomplish this by lowering gastrointestinal cholesterol absorption. Recent studies report that vytorin and zetia, two major synthetic drugs administered in the treatment of high cholesterol levels failed to decrease the incidence of heart disease (Mitka, 2008). On the other hand, the use of anti-obesity drugs is severely restricted due to the accompanying side effects (Wasan and Looije, 2005). Hence, there is need to explore more efficient, safe and economic alternatives to combat dyslipidemia and associated metabolic disorders.

Saponin extracts from *E. elephantina* and *P. prunelloides* may also be exploited as hypocholesterolemic as well as antioxidative alternatives to the synthetic drugs that are implicated for a number of side effects. The binding of saponins to bile acids may have important implications in the mitigation of carcinogenesis. Bile acids excreted in the liver are termed primary bile acids. They are metabolized in the colon thereby producing secondary bile acids (Pollak et al., 1985). Some of the so formed secondary bile acids promote colon cancer. By binding to primary bile acids resulting in the formation of phytosomes, it may be proposed that saponins from *E.*

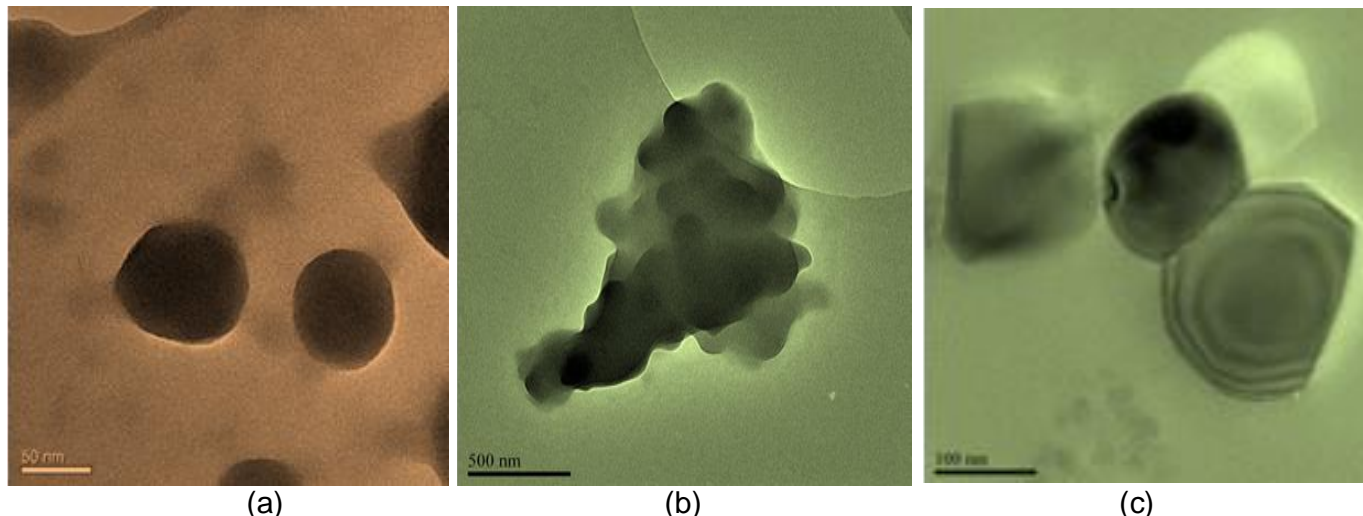


Figure 6. TEM saponin + cholesterol + L- α -phosphatidylcholine (freshly prepared).

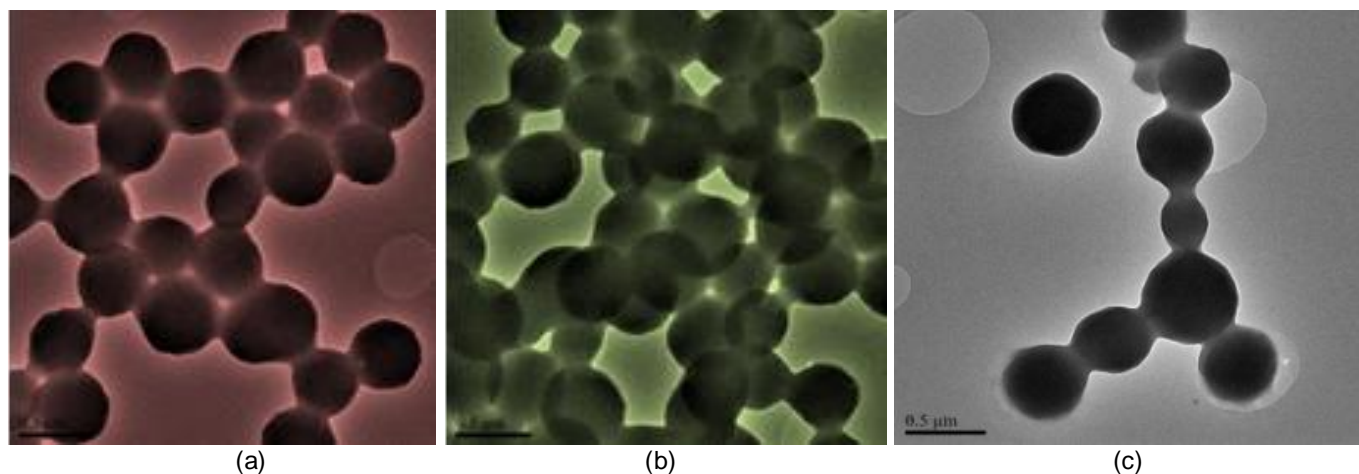


Figure 7. TEM saponin + cholesterol + L- α -phosphatidylcholine (after 3 weeks).

elephantina and *P. prunelloides* may reduce the formation of the secondary bile acids; hence, reducing chances of colon cancer. A variety of natural products (Pollak, 1985; Price et al., 1987; Malinow, 1984; Miettinen et al., 1976; Cayen 1971) have been shown to inhibit cholesterol absorption from intestinal lumen in experimental animals, and consequently reduce the concentration of cholesterol in the plasma. Although, there is controversy on the mechanism of cholesterol reduction by saponins, there is a consensus on its ultimate reduction (Temel et al., 2009).

Mortality from cardiovascular disease is ranked the second highest cause of death worldwide (Malach and Imperato, 2006). This anomaly is generally attributed to the elevated levels of plasma cholesterol (hypercholesterolemia) which results in coronary heart disease (Altman, 2003; Jalali-khanabadi et al., 2006). Elevated lipid levels

can be decreased by controlled diet to avert hyperlipidemia. The use of anti-hyperlipidemic drugs is more practical but these are not always satisfactory due to the side effects of chemically synthesized drugs (Chiang et al., 2007). Phytopharmaceuticals are gaining importance in allopathic as well as traditional medicine due to their non-additive and non-toxic nature (Jenkins et al., 1983; Raskin et al., 2002). The presence of a significant amount of saponins in both *E. elephantina* and *P. prunelloides* lends support to the wide application of these two medicinal plants for the various ailments for which they are administered in southern Africa. Diosgenin that was also identified in both *P. prunelloides* and *E. elephantina* has been implicated for having favourable effects on glucose lowering (McAnuff et al., 2005) and antioxidant activity (Son et al., 2007; Jayachandra et al., 2009).

Conclusion

This study demonstrates for the first time that *P. prunelloides* and *E. elephantina* saponin extracts and fractions have the potential to bind cholesterol *in vitro* resulting in the formation of phytosomes with particle sizes ranging between tens and hundreds nanometers. Spherical nanoparticles/phytosomes/liposomes were clearly identified in the TEM images with particle sizes ranging between 10 and 500 nm. The zeta potential for the particles characterized by the 'zeta sizer' fell between -5 and +5 mV. This clearly demonstrates that the particles so formed have a high degree of aggregation, a characteristic feature that may be proposed for the reduction of the gastrointestinal absorption of saponins. Since *P. prunelloides* and *E. elephantina* exhibited a high triterpenoid saponin content, these two medicinal plants could be used as alternative sources of saponins in the manufacture of adjuvants or phytosomes in place of synthetic as well as Quillaja saponins that have their shortcomings.

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Full Length Research Paper

Optimization of chromium biosorption in aqueous solution by marine yeast biomass of *Yarrowia lipolytica* using Doehlert experimental design

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Optimization of a chromium biosorption process was performed by varying three independent variables pH (0.5 to 3.5), initial chromium ion concentration (10 to 30 mg/L), and *Yarrowia lipolytica* dosage (2 to 4 g/L) using a Doehlert experimental design (DD) involving response surface methodology (RSM). For the maximum biosorption of chromium ion in an aqueous solution by *Y. lipolytica*, a total of fifteen experimental runs were set and the experimental data fitted to the empirical second-order polynomial model of a suitable degree. The analysis of variance of the quadratic model demonstrates that the model was highly significant. The model showed that chromium uptake in aqueous solution was affected by all the three variables studied. The optimum values of the variables were found to be 2.07, 18.76 mg/L and 3.39 g/L for pH, initial chromium ion concentration and biomass dosage, respectively at contact time of 30 min. At these optimal conditions, the maximum percentage biosorption of chromium was predicted to be 41.59. The experimental values were in good agreement with predicted values and the correlation coefficient was found to be 0.9891. Therefore, it is apparent that the DD involving RSM not only gives valuable information on interactions between the variables but also leads to identification of feasible optimum values of the studied variables.

Key words: Biosorption, Doehlert experimental design, response surface methodology, *Yarrowia lipolytica*.

INTRODUCTION

The presence of toxic heavy metals contaminated in aqueous streams, arising from the discharge of untreated metal containing effluent into water bodies, is one of the

most important environmental issues (Hawari and Mulligan, 2006). Their presence in aquatic ecosystem causes harmful effect to living organisms (Xuejiang et al.,

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2006). In recent years, increased attention has focused on the use of microorganisms for the removal and possible recovery of metal ions from industrial wastes. In fact, it represents a potential alternative to existing technologies (chemical precipitation, reverse osmosis, solvent extraction), which have significant disadvantages such as high reagent or energy requirements and generation of toxic sludge or other products that need disposal (Bossrez et al., 1997). Biosorption, the passive binding of metals by living or dead biomass, can be used to remove toxic heavy metals from industrial wastewater. Chromium and its compounds are ubiquitous and persistent environmental contaminants released into natural environment from a variety of anthropogenic sources, including the electroplating, leather tanning processes, chromite ore processing, wood preservation, alloys making, corrosion control, pigment and dyes, and metal finishing industries (Suksabye et al., 2008; Quintelas et al., 2009). Though the removal of Cr from wastewater is obligatory before discharging into aquatic environment, the rest in the effluent will still cause serious environmental impact. Therefore, the discharge of Cr into aquatic ecosystems has become a matter of concern over the last few decades (Massara et al., 2008; Suksabye et al., 2008; Ye et al., 2010). Strong exposure of Cr (VI) causes cancer in digestive tract and lungs and may cause epigastric pain, nausea, vomiting, severe diarrhea and hemorrhage (Mohanty et al., 2006). It is therefore very urgent to control chromium contamination. Considerable efforts have thus been devoted to developing available technologies which can remove chromium from the effluents of those industries.

In recent years, *Yarrowia lipolytica* has emerged as important non-conventional yeast with significant biological relevance and biotechnological applications (Barth and Gaillardin, 1997; Fickers et al., 2005). This yeast has been used in the remediation of various polluted environments (Margesin and Schinner, 1997; Zinjarde and Pant, 2002; Jain et al., 2004) and is also applied in the degradation of different wastes (Johnson et al., 1994; De Felice et al., 1997; Oswal et al., 2002; Lanciotti et al., 2005). *Y. lipolytica* is able to utilize a variety of renewable carbon sources and the biomass of the fungus has been used as single cell protein or as single cell oil (Achremowicz et al., 1977; Papanikolaou et al., 2002). However, there are few reports available regarding how this yeast can survive metal stress and accumulate different metals (García et al., 2002; Strouhal et al., 2003; Ito et al., 2007; Agnihotri et al., 2009; Bankar et al., 2009). This microorganism therefore, displays a potential for the bioremediation of metal polluted environments. Biosorption of chromium ions by different living and nonliving biomass have been studied by several authors (Aksu and Balibek, 2007; Yin et al., 2008; Aksu et al., 2009; Khambhaty et al., 2009; Ye et al., 2010).

Optimization of biosorption of heavy metals by the

classical method involves changing one independent variable (that is *Y. lipolytica* dosages, pH, heavy metal concentration, temperature) while maintaining all others at a fixed level which is extremely time consuming and expensive for a large number of variables. These drawbacks of single parameter optimization process can be eliminated by optimizing all the affecting parameters collectively by Doehlert experimental design (DD) (Doehlert, 1970) of Response Surface Methodology (RSM). Basically, this optimization process involves three major steps: performing the statistically designed experiments, estimating the coefficients in a mathematical model, predicting the response and checking the adequacy of the model. Several researchers have applied various designs for optimization of different process parameters (Preetha and Viruthagiri, 2007; Kumar et al., 2009; Kiran and Thanasekaran, 2011; Mona et al., 2011; Bermúdez et al., 2012). The objective of the present study was to optimize biosorption of chromium (VI) ions in aqueous solution onto *Y. lipolytica* in a batch experiment. For better understanding of different stages of biosorption at varying heavy metal concentration, pH and sorbent dosages, RSM was used to optimize heavy metal uptake.

MATERIALS AND METHODS

Microorganism and growth conditions

Yarrowia lipolytica NCIM 3589, obtained from National Chemical Laboratory, Pune, India, was used throughout the study. The culture was maintained on MGYB slants having the composition (%): malt extract 0.3, glucose 1.0, yeast extract 0.3, peptone 0.5 and agar agar 2.0. The pH of the medium was adjusted to 6.4 to 6.8 and culture was incubated at 30°C for 48 h. Subculturing was carried out once in two weeks and the culture was stored at 4°C.

Microbial cultivation

The composition of culture medium was (grams per liter): peptone: 5; yeast extract: 3 and NaCl: 3 (Imandi et al., 2008). The medium was sterilized by autoclaving at a pressure of 1.5 atm and temperature of 121°C for 20 min. The yeast cells were grown for 16 h (at end of exponential phase) and then filtered (Imandi et al., 2010).

Preparation of biosorbent

Yeast biomass was deactivated by heating in an oven at 80°C for 24 h (Schiewer and Volesky, 1995). The dried yeast was ground and screened through a sieve with 100 mesh. The pretreatment of the biosorbent was carried out with nonviable yeast cells in 700 g/L ethanol solution for 20 min at room temperature. Then, it was centrifuged at 3600 rpm for 10 min and the ethanol solution was discarded. The ethanol washed biomass was rinsed several times with deionized water to remove excess ethanol and adsorbed nutrient ions. The rinsed yeast was again centrifuged and the remaining biomass was dried at 70°C for 12 h (Göksungur et al., 2005). The dried cells were ground and screened as mentioned above. The purpose of grinding dried yeast was to make a

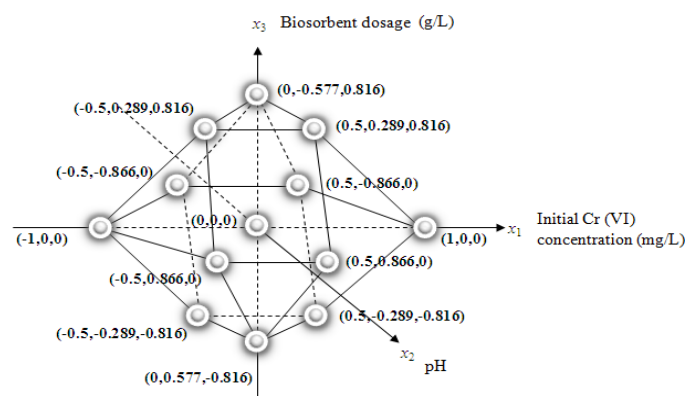


Figure 1. 3D view of the experimental domain. The dots represent the experimental runs of the Table 2.

homogenized yeast biomass in order to destroy biomass aggregates and increase uptake capacity (Bahadir et al., 2007).

Preparation of chromium solution

All the chemicals used were of analytical grade. Stock solution of 1000 mg/L was prepared by dissolving appropriate amount of $K_2Cr_2O_7$ in 1 L of deionized water. The pH of the metal solution was adjusted to a desired value with 0.1 N NaOH and 0.1 N HCL. Chromium solution of different concentrations was prepared by suitable dilution of the stock solution to known volumes.

Experimental procedure

2 g/L of biosorbent was added to 50 mL of the chromium ion solution in each of 250 mL Erlenmeyer flasks. The flasks were incubated in orbital shaker at a speed of 180 rpm for different agitation times (1, 2, 5, 10, 20, 30, 40, 50, 60, 90 and 120 min). The resulting samples were filtered by Whatman filter paper and analyzed for chromium concentration in flame atomic absorption spectrometer (novAA[®] 350, Analytikjena, Germany).

$$\text{The percent chromium biosorbed} = \frac{C_o - C_i}{C_o} \times 100$$

is calculated from the relation

(1)

Where, C_o is the initial concentration of chromium in the aqueous solution (mg/L); C_i is the final concentration of chromium in the aqueous solution (mg/ L).

Preliminary experimental runs for the biosorption of chromium using *Yarrowia lipolytica* has been carried out by assigning three experimental strategies, as listed below, varying one parameter and keeping the other two parameters constant for an equilibrium agitation time and temperature at 303 K.

- Strategy 1: pH was varied as 1, 2, 3, 4, 6 and 7 keeping the biosorbent dosage and initial chromium ion concentration at constant values.
- Strategy 2: Initial chromium ion concentration was varied as (mg/L) 20, 50, 100, 150 and 200, keeping the other parameters constant.
- Strategy 3: Keeping pH and initial chromium ion concentration as constants, the biosorbent dosage was varied as (g/L) 1, 2, 3, 4 and 5.

Doehlert experimental design

Once the variables having the statistically significant influence on the responses were identified, a Doehlert experimental design (Doehlert, 1970) was used to optimize the values of these variables. The number of experiments required (N) is given by $N = n^2 + n + n_0$, where, n is the number of variables and n_0 is the number of center points. Replicates at the central level of the variables were performed in order to validate the model by means of an estimate of experimental variance. For statistical calculations, the natural variables X_i were coded as x_i according to Equation (2)

$$x_i = \left(\frac{X_i - X_{oi}}{\Delta X_i} \right) \alpha_i \quad (2)$$

Where, x_i is the coded value of the i^{th} variable, X_i the natural value, X_{oi} the value at the center point, ΔX_i the step change value, and α_i is the maximum value of the coded variable (that is 1.0, 0.866 and 0.816 for five levels, seven levels, and three levels, respectively).

The second degree polynomial (Equation 3) was fitted to the experimental data by using the statistical software STATISTICA 6.0 (Stat-Ease Inc., Tulsa, OK, USA) to estimate the response of the dependent variable and the regression coefficients.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{13}X_1X_3 \quad (3)$$

Where, Y is the predicted response; X_1, X_2, X_3 are the independent variables; b_0 is the offset term; b_1, b_2, b_3 are the coefficients for linear effects, b_{11}, b_{22}, b_{33} are the coefficients for squared effects and b_{12}, b_{23}, b_{13} are the coefficients for interaction terms.

Doehlert design offers the following advantages over the other designs of RSM. Firstly, this design needs fewer experiments; secondly, the number of levels is not the same for all variables, which allows flexibility to assign a large or a small number of levels to the selected variables depending on their relative importance. Generally it is preferable to choose the variable with the stronger effect as the variable with seven levels in order to obtain maximum information of the system. Thirdly, considering that the efficiency of any experimental design is defined as the number of coefficients of the model divided by the number of experiments, Doehlert design is more efficient than Central Composite design or Box-Behnken design. Fourthly, Doehlert design is also more efficient in mapping the space: adjoining hexagons can fill a space completely and efficiently, since the hexagons fill space without overlap (Imandi et al., 2007).

Figure 1 shows the graphical representation of this network in the space defined by the factors. This uniform distribution of experimental points (shown as dots) allows interpolation by the mathematical model of responses anywhere within the experimental domain. Furthermore, the quality of the interpolation remains constant since the network is uniform. The Doehlert design shows great flexibility compared to other classical designs used in the process optimization.

RESULTS AND DISCUSSION

Effect of contact time

Time course profile for the biosorption of Cr (VI) for a solution of 20 mg/L is shown in Figure 2. The data showed that a contact time of 30 min was required to achieve an

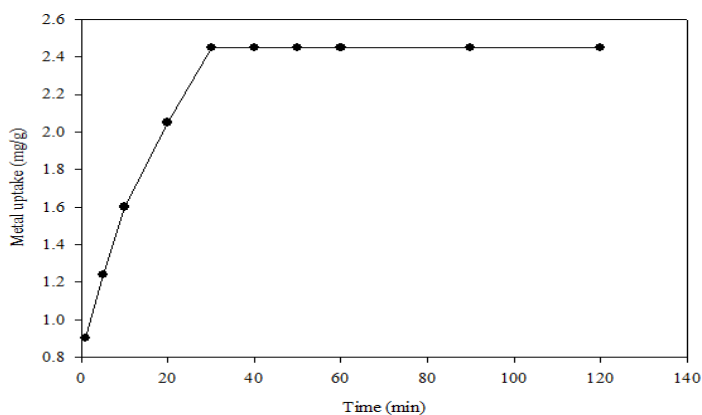


Figure 2. Effect of contact time on biosorption of chromium by *Y. lipolytica*.

optimum biosorption and there were no significant change in the removal of metal ions with further increase in contact time. Therefore, the uptake and unabsorbed chromium concentrations at the end of 30 min are given as the equilibrium values, q_{eq} (mg/g) and C_{eq} (mg/L). For further studies of biosorption with other variable parameters the optimum time of 30 min for Cr (VI) was chosen for contact period.

Effect of pH

Earlier studies on heavy metal biosorption has shown that pH of the solution is most important physical parameter influencing the adsorption process. Batch biosorption experiments are conducted at different pH (1.0 to 7.0) values and the results are depicted in Figure 3. The biosorption capacity for the *Y. lipolytica* was greater at pH 2.0 when compared to higher values of pH (Figure 3). All subsequent experiments were therefore carried out at pH 2.0. The results also suggest that active processes displayed by live cells may not be involved in biosorption of Cr (VI). Such observations on enhanced biosorption at acidic pH has been previously reported with other biosorbents such as bacteria (Zhou et al., 2007), fungi (Özer and Özer, 2003; Sen et al., 2005), milled peat (Dean and Tobin, 1999), cone biomass (Ucun et al., 2002), pods, leaves and bark of an ornamental plant (Abbas et al., 2008) and the husk of Bengal gram (Ahalya et al., 2005). The reason for the enhanced adsorption of Cr (VI) at low pH was that negatively charged $[\text{HCrO}_4]^-$, $[\text{Cr}_2\text{O}_7]^{2-}$, $[\text{Cr}_4\text{O}_{13}]^{2-}$ and $[\text{Cr}_3\text{O}_{10}]^{2-}$ ions are the dominant species under such conditions. The surfaces of yeast cell walls at low pH are surrounded by hydronium ions (H_3O^+). The negatively charged ion species are thus effectively adsorbed on the positively charged active sites on the sorbent (Özer and Özer, 2003). With an increase in pH, the binding of ions decreased on account of repulsive

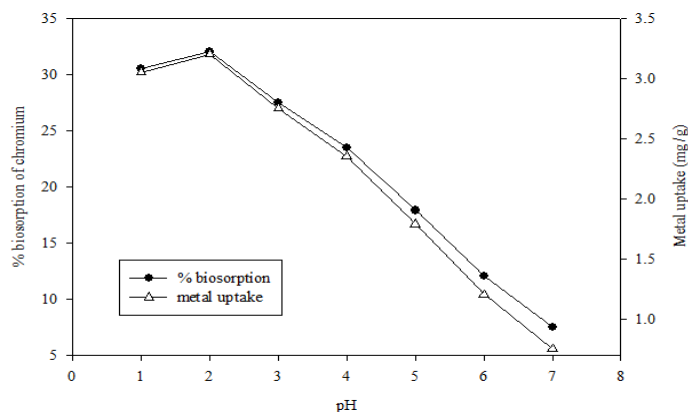


Figure 3. Effect of pH on chromium biosorption for 20 mg/L of metal and 2 g/L of biomass concentration.

forces between the biosorbent (*Y. lipolytica*) and Cr (VI) ions. It is therefore proposed that in a manner similar to *Bacillus thuringiensis*, in *Y. lipolytica* also, the interactions may be primarily electrostatic or coordinative in nature (Şahin and Öztürk, 2005).

Effect of initial chromium ion concentration

The relationship between the percentage biosorption of Cr (VI) and its initial concentrations using different dosages of yeast biomass are presented in Figure 4. The initial metal ion concentration remarkably influenced the equilibrium metal uptake and biosorption yield. The amount of metal ion adsorbed increased with increase in initial concentrations (due to higher availability of metal ions for sorption) while the percentage biosorption of metal ion decreases with an increase in initial metal ion concentration. The increase of metal uptake is a result of the increase in the driving force, that is concentration gradient, with an increase in the initial metal ion concentrations (from 20 to 200 mg/L). However, the percentage biosorption of Cr (VI) on yeast biomass decreased from 32 to 23%, respectively. Though an increase in metal uptake was observed, the decrease in percentage biosorption may be attributed to lack of sufficient surface area to accommodate much more metal available in the solution. At lower concentrations, all metal ions present in solution could interact with the binding sites and thus the percentage biosorption were higher than those at higher initial metal ion concentrations. At higher concentrations, lower biosorption yield is due to the saturation of biosorption sites.

Effect of biosorbent dosage

Studies on the effect of yeast biomass dosage for Cr (VI) removal is important to get the trade-off between the

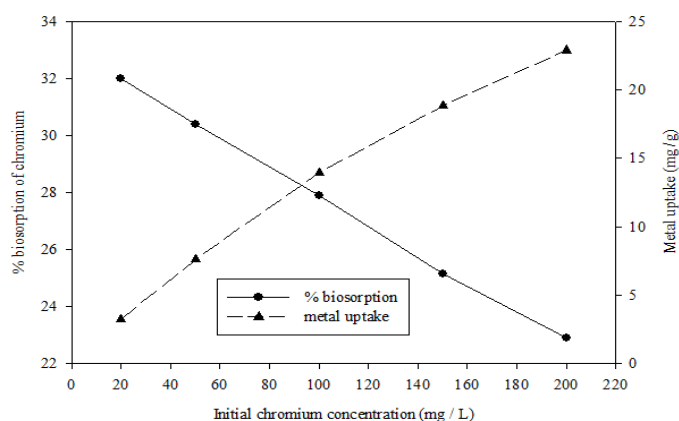


Figure 4. Effect of Cr (VI) ion concentrations on biosorption of chromium by *Y. lipolytica* at 2 g/L of biosorbent concentration (pH 2).

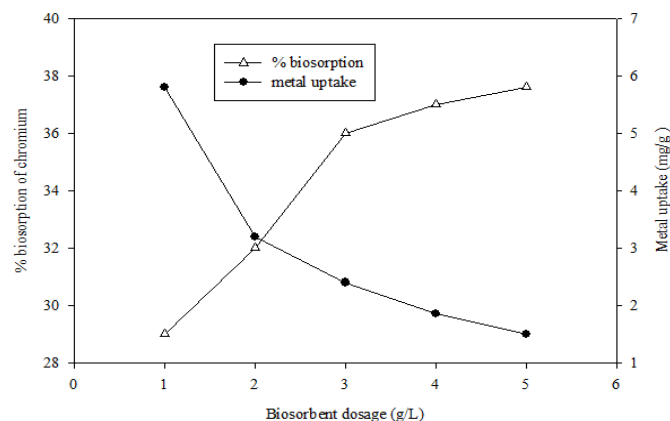


Figure 5. Effect of biosorbent dosage on biosorption of chromium for 20 mg/L of metal concentration (pH 2).

adsorbent capacity and percent removal of metal ions resulting in an optimum biosorbent concentration. The percent of biosorption and adsorption capacity for Cr (VI) as a function of biosorbent dosage was investigated (Figure 5). The removal of chromium was also dependent on the concentration of biomass used in the biosorption medium. The result shows that with the increase in the yeast biomass dosage, the metal removal efficiency also increased. The percentage biosorption of chromium increased 29 to 37%. Results show that the biosorption efficiency is highly dependent on the increase in biomass dosage of the solution. This is expected because higher dosage of adsorbent in the solution, increase the availability of exchangeable sites for the ions. It was earlier reported that the increase in the efficiency of metal ions removal with an increase in the biosorbent dosage was due to the increase in the number of adsorption sites (Vinod et al., 2010). The drop in adsorption capacity is basically due to the sites remaining unsaturated during the adsorption process.

Optimization of the process variables using DD

The selection of the range for process variables is extremely important when planning the experimental design; otherwise, after completion of the experimental runs, the optimal conditions, obtained either by response surface methodology, might not be found inside the experimental region. The following experiments were carried out to study the variables in such a range so that reasonable percentage removal of chromium would be achieved within that range. From the results of preliminary experimental runs, the three variables (initial chromium concentration, pH, and biosorbent dosage) have been identified as the potential variables for the percentage biosorption of chromium. Out of them, the pH had shown stronger effect on percentage biosorption of

chromium and hence it was assigned seven level, followed by initial chromium concentration assigned five level and biosorbent dosage assigned three levels. A summary of the independent variables and their range and levels is presented in Table 1.

Fifteen (15) experimental runs (Table 2) including three replicates at the center point were carried out for 30 min of contact time. By using multiple regression analysis (STATISTICA 6.0) the coefficients of equation (4) was estimated, and gave the following equation.

$$Y = - 61.7717 + 3.0454X_1 + 27.5904X_2 + 27.6128X_3 - 0.0846X_1^2 - 4.6417X_2^2 - 3.4006X_3^2 - 0.0137X_1X_2 - 2.7006X_2X_3 + 0.0468X_1X_3 \quad (4)$$

The predicted percentage biosorption of chromium resulted from equation (4) are in close agreement with the experimental values as evident from the last column of Table 2, and hence the above equation was deemed to be adequate in representing the percentage biosorption of chromium under the specified range of experiments. For quadratic models, the optimum point can be characterized as maximum, minimum, or saddle. It is possible to calculate the coordinates of the optimum point through the first derivate of the mathematical function, which describes the response surface and equates it to zero. The above quadratic equation obtained for three variables as described below:

$$\frac{\partial Y}{\partial X_1} = 3.0454 - 0.1692 X_1 - 0.0137 X_2 + 0.0468 X_3 = 0 \quad (5)$$

$$\frac{\partial Y}{\partial X_2} = 27.5904 - 0.0137 X_1 - 9.2834 X_2 - 2.7006 X_3 = 0 \quad (6)$$

$$\frac{\partial Y}{\partial X_3} = 27.6128 + 0.0468 X_1 - 2.7006 X_2 - 6.8012 X_3 = 0 \quad (7)$$

Table 1. Experimental range and levels of the variables.

Variable	Range and level						
Coded variable, x_1	-1	-0.5	0	0.5	1		
Initial chromium concentration, X_1 (mg/L)	10	15	20	25	30		
Coded variable, x_2	-0.866	-0.577	-0.288	0	0.288	0.577	0.866
pH, X_2	0.5	1.0	1.5	2.0	2.5	3.0	3.5
Coded variable, x_3	-0.816	0	0.816				
Biosorbent dosage, X_3 (g/L)	2.0	3.0	4.0				

Table 2. Doehlert three variable experimental design along with experimental and predicted values.

Experiment number	Coded value			Natural value			% biosorption of chromium	
	x_1	x_2	x_3	X_1	X_2	X_3	Experimental	Predicted
1	1	0	0	30	2.0	3.0	29.26	30.3487
2	-1	0	0	10	2.0	3.0	34.21	34.1212
3	0.5	0.866	0	25	3.5	3.0	28.42	27.8775
4	-0.5	-0.866	0	15	0.5	3.0	26.65	28.1925
5	0.5	-0.866	0	25	0.5	3.0	25.35	26.5112
6	-0.5	0.866	0	15	3.5	3.0	30.13	29.9687
7	0.5	0.288	0.816	25	2.5	4.0	33.48	32.9337
8	-0.5	-0.288	-0.816	15	1.5	2.0	31.26	29.8062
9	0.5	-0.288	-0.816	25	1.5	2.0	28.27	29.0200
10	0	0.577	-0.816	20	3.0	2.0	31.43	33.1337
11	-0.5	0.288	0.816	15	2.5	4.0	35.67	35.9200
12	0	-0.577	0.816	20	1.0	4.0	38.28	36.5762
13	0	0	0	20	2.0	3.0	40.15	40.1966
14	0	0	0	20	2.0	3.0	40.32	40.1966
15	0	0	0	20	2.0	3.0	40.12	40.1966

X_1 , Initial chromium concentration (mg/L); X_2 , pH, X_3 , biosorbent dosage (g/L).

Thus, to calculate the coordinate of the optimum point, it is necessary to solve the first grade system formed by Equations. (5), (6) and (7) and to find the $X_{1, \text{opt}}$, $X_{2, \text{opt}}$ and dosage = 3.39 g/L. The extent of biosorption of chromium at these optimum conditions was 41.59%.

The significance of each coefficient in equation (4) was determined by student's t -test and p -values which were also listed in Table 3. The larger the magnitude of the t -value and smaller the p -value, the more significant is the corresponding coefficient (Lazić, 2004). This implies that the linear and quadratic effects of initial chromium concentration, pH and biosorbent dosage were highly X_3 , opt values. The optimal set of conditions for maximum percentage biosorption of chromium is pH = 2.07, initial chromium concentration = 18.76 mg/L, and biosorbent significant as is evident from their respective p -values. The interaction effect of pH and biosorbent dosage was found to be significant ($p \leq 0.05$). The remaining two interaction terms that is, initial chromium concentration \times pH

and biosorbent dosage \times initial chromium concentration were found to be insignificant ($p > 0.05$) which were also presented in Table 3. The parity plot (Figure 6) showed a satisfactory correlation between the experimental and predicted values of percentage biosorption of chromium, wherein, the points cluster around the diagonal line which indicated the good fit of the model because the deviation between the experimental and predicted values is less.

The results of the second order response surface model fitting in the form of Analysis of Variance (ANOVA) are given in Table 4. It is required to test the significance and adequacy of the model. The Fisher variance ratio, the F -value ($= S_r^2 / S_e^2$), is a statistically valid measure of how well the factors describe the variation in the data about its mean. The greater the F -value is from unity, the more certain it is that the factors explain adequately the variation in the data about its mean, and the estimated factor effects are real. The ANOVA of the regression model demonstrates that the model is highly significant,

Table 3. Model coefficients estimated by multiple linear regression (significance of regression coefficients).

Term	Coefficient	Value	Standard error of coefficient	t-value	p-value
Constant	b_0	-61.7717	12.62456	-4.8930	0.004502*
Initial Cr (VI) concentration	b_1	3.0454	0.59717	5.0996	0.003771*
pH	b_2	27.5904	3.44779	8.0023	0.000492*
Biosorbent dosage	b_3	27.6128	5.33202	5.1787	0.003529*
Initial Cr (VI) concentration × initial Cr (VI) concentration	b_{11}	-0.0846	0.01144	-7.3944	0.000712*
pH × pH	b_{22}	-4.6417	0.38144	-12.1687	0.000066*
Biosorbent dosage × biosorbent dosage	b_{33}	-3.4006	0.72374	-4.6986	0.005344*
Initial Cr (VI) concentration × pH	b_{12}	-0.0137	0.08357	-0.1635	0.876502
pH × biosorbent dosage	b_{23}	-2.7006	0.76289	-3.5399	0.016564*
Biosorbent dosage × initial Cr (VI) concentration	b_{31}	0.0468	0.13214	0.3544	0.737472

*Significant at $p \leq 0.05$.

Table 4. ANOVA for the entire quadratic model.

Source of variation	Sum of square (SS)	Degree of freedom (d.f.)	Mean square (MS)	F-value	Probe>F
Model	353.8888	9	39.32098	25.02308	0.001226
Error	7.8569	5	1.57139		
Total	361.7457	14			

$R = 0.98908063$; $R^2 = 0.97828049$; Adjusted $R^2 = 0.93918536$. $P_{\text{model}} > F = 0.001226$.

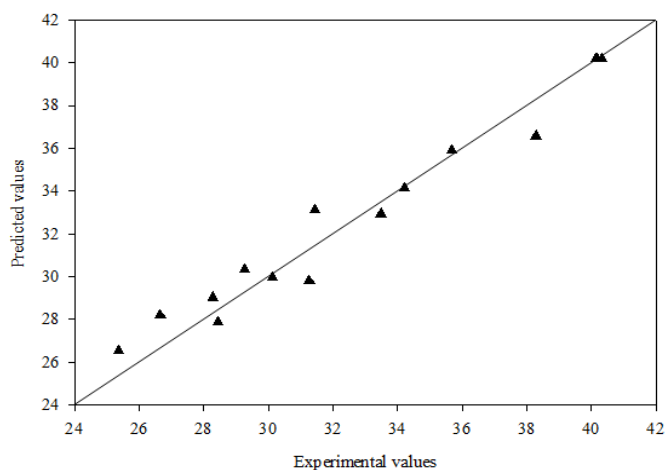


Figure 6. Parity plot showing the distribution of experimental vs. predicted values of percentage biosorption of chromium.

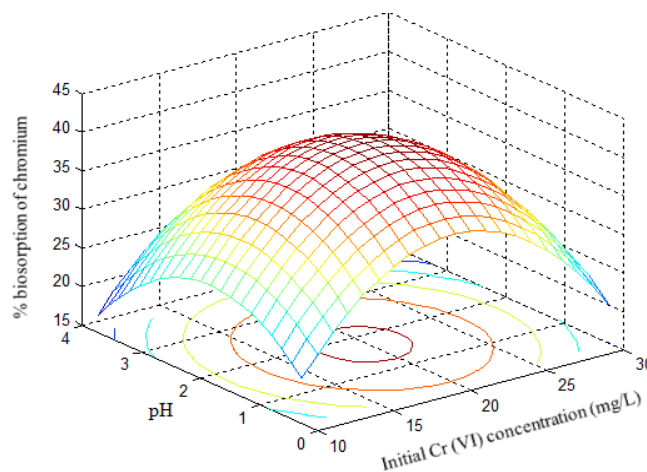


Figure 7. Response surface and contour plot of initial chromium concentration vs. pH on percentage biosorption of chromium (biosorbent dosage was kept constant at 3 g/L).

as is evident from the Fisher's F -test ($F_{\text{model}} = 25.02308$) and a very low probability value ($P_{\text{model}} > F = 0.001226$). The goodness of fit of the model was checked by the determination coefficient (R^2). The R^2 value provides a measure of how much variability in the observed

response values can be explained by the experimental variables and their interactions. The R^2 value is always between 0 and 1. The closer the R^2 value is to 1, the stronger the model is and the better it predicts the

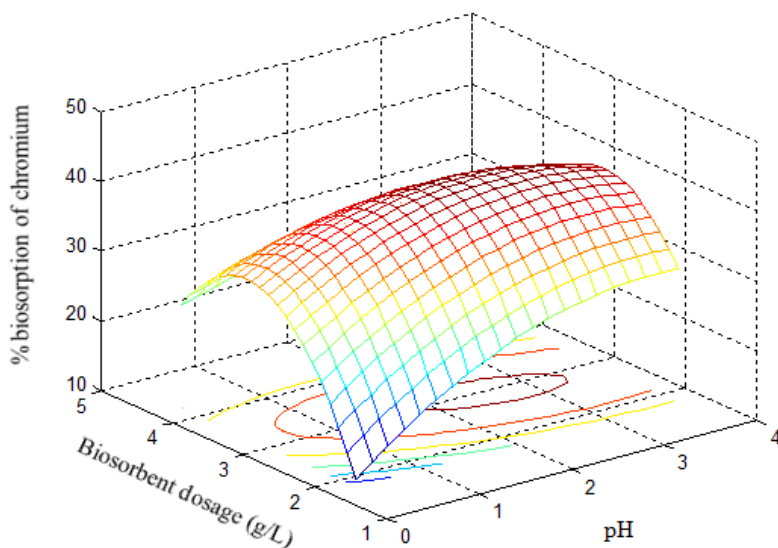


Figure 8. Response surface and contour plot of pH vs. biosorbent dosage on percentage biosorption of chromium (initial Cr (VI) concentration was kept constant at 20 mg/L).

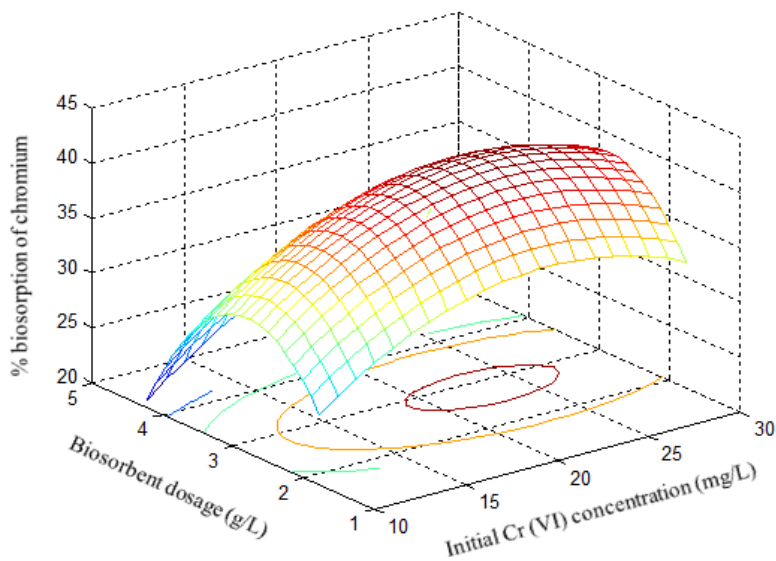


Figure 9. Response surface and contour plot of biosorbent dosage vs. initial chromium concentration on percentage biosorption of chromium (pH was kept constant at 2).

response. In this case, the value of the determination coefficient ($R^2 = 0.9783$) indicates that 97.83 % of the variability in the response could be explained by the model. In addition, the value of the adjusted determination coefficient ($Adj R^2 = 0.9392$) is also very high to advocate for a high significance of the model. Also a higher value of the correlation coefficient ($R=0.9891$) justifies an excellent correlation between the independent process variables. The response surface

contour plots of percentage biosorption of chromium versus the interactive effect of pH, initial chromium concentration, and biosorbent dosage are shown in the Figures 7 to 9.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Role of c-Src inhibitor in the regulation of hepatocarcinoma cell migration

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It has been discovered that hepatocellular carcinoma (HCC) has high ability of migration and angiogenesis. This study aimed to explore the mechanism of HCC cell migration and angiogenesis. BEL-7402 cell line was used as HCC cell model for investigating the regulation of cell migration upon c-Src inhibitors (PP2 and SU6656) treatment. Western blot was used for detecting the expression of MT1-MMP and VEGF-C. The activity of MMP2 and MMP9 was monitored with gelatin zymography assay. BEL-7402 cell migration and invasion was detected by wound healing assay and Transwell. Immunoprecipitation was used for detecting the interaction among c-Src, pro-MT1-MMP, Furin and VEGF-C. Our results have show that the expression of MT1-MMP and VEGF-C were inhibited by PP2 and SU6656, in accordance with c-Src activity. Zymography assay demonstrated that the activity of MMP2 and MMP9 decreased upon PP2 or SU6656 treatment. The invasion and migration of BEL-7402 were inhibited. We also found that c-Src interacted with Furin *in vivo*. The interaction between Furin and its substrates pro-MT1-MMP, pro-VEGF-C decreased upon c-Src inhibitors treatment. These findings indicate that the activity of c-Src inhibition associated with cell invasion and migration decreased by down-regulating the interaction between Furin and its substrates (pro-MT1-MMP, pro-VEGF-C).

Key words: Hepatocellular carcinoma (HCC), Furin, c-Src inhibitor, MT1-MMP, VEGF-C, cell migration.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the leading cause of cancer-related death in the world, which has a high potential ability of metastasis in tumor progression (GLOBOCAN, 2008). However, the underlying mechanisms of HCC initiation, progression and metastasis are still not fully understood (Tang et al., 2010). Cell migration related protein MT1-MMP, MMP2, VEGF must be cleaved by protein convertase, followed by maturation

and activation.

Furin is the best-characterized representative of the mammalian subtilisin-like family of proprotein convertase. It is synthesized as inactive proenzyme and rapidly matured by autocatalytic cleavage between the prodomain and the catalytic domain in endoplasmic reticulum (ER) (Vey et al., 1994). Following this initial cleavage, the propeptide-Furin complex leaves the ER and enters the

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trans-golgi network TGN) for its second cleavage (Anderson et al., 1997). Hence, Furin becomes active to process substrate molecules in multiple compartments in the TGN/endosomal system (Molloy et al., 1994). Many protein precursors such as matrix metalloproteases, hormones, growth factors, serum proteins, receptors, and adhesion molecules have identified the Furin substrates (Fujisawa et al., 2004; Louagie et al., 2008; Yana and Weiss, 2000). MT1-MMP proenzyme cleavage by Furin is considered to be a principal event in the activation of this substrate and it may be important in HCC cell migration and invasion (Dangi-Garimella et al., 2011).

Furin activation plays a vital role in tumor process (Lopez de Cicco et al., 2005). Furin inhibitor α 1-PDX has been used to block Furin activity and to prevent cancer metastasis in biochemical, cellular and animal studies (Molloy and Thomas, 2001). As the most closely related members of the Src family of nonreceptor tyrosine kinases, it up-regulates c-Src correlates with a variety of human tumors, including cancer of the HCC (Hilbig, 2008; Ischenko et al., 2007). c-Src and Furin have been found to be upregulated in human cancer, but the ubiquitous c-Src participating in the interaction between Furin and substrates still remains unknown.

Stawowy et al. (2002) have demonstrated that Furin-like proprotein convertase PC5 was strongly upregulated by platelet derived growth factor-BB (PDGF-BB) through PI3-kinase/p70s6-kinase pathway (Philipp et al., 2002). A similar mechanism may also apply to the convertase Furin. We then investigate whether Furin is regulated by PDGF-BB through c-Src kinase and how Furin activity is controlled to mediate the processing of its substrate MT1-MMP and VEGF-C.

In this study, we detected the protein level and the interaction between Furin and its substrates stimulated with PDGF-BB 30 min after pretreatment with c-Src inhibitor PP2 or SU6656 in HCC cells. In this short article, we will attempt to understand the new pathogenesis that activation of Furin is c-Src dependent in HCC cells and has a promising strategy against HCC metastasis.

MATERIALS AND METHODS

Cell culture and experimental reagents

HCC cell lines BEL-7402 were cultured in RPMI 1640 *invitrogen* supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μ g/ml streptomycin. All cells were cultured in a 5% CO₂ humidified atmosphere at 37°C. In some experiments, cells were cultured in serum-free medium wherever indicated. PDGF-BB (20 ng/ml), research and development (R&D, USA) or c-Src inhibitors PP2 or SU6656 (10 μ M) were added to the medium whenever necessary as indicated in the figure legend.

Primary antibodies against pSrc^{Y416}, Furin, MT1-MMP, VEGF-C and β -actin were purchased from Santa Cruz Biotechnology Santa Cruz, USA. Gelatin Zymography kit Millipore (USA), 4-amino-5-(4-chlorophenyl)-7-t-butyl) pyrazolo (3,4-d) pyrimidine PP2) and PDGF-BB were purchased from Enzo Life Sciences International, USA; SU6656 Sigma (USA).

Western blot analysis

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer of 50 mM Tris with pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulphate (SDS), 1% sodium deoxycholate, 5 mM ethylenediaminetetraacetic acid (EDTA), 100 mM NaF, and 1 mM Na₃VO₄ containing protease inhibitor cocktail for 30 minutes at 4°C. All cell lysates (16,000 g) were centrifuged at 4°C for 30 min. The protein concentration was determined with the pierce bicinchoninic acid (BCA) method (USA). Aliquots of cell lysates were fractionated by electrophoresis in sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE): 8% for the analysis of Furin and c-Src, 10% for the analysis of MT1-MMP and VEGF-C. Total proteins were electroblotted to polyvinylidene fluoride (PVDF) membrane using a wet transblot system Bio-Rad, Hercules (CA). Blots were then blocked for 1 h at room temperature with 10% bovine serum albumin (BSA) or 5% nonfat dry milk. Membranes were incubated overnight at 4°C with antibodies against pSrc^{Y416}, Furin, MT1-MMP, VEGF-C and β -actin (1:1000). After subsequent washing, the membranes were incubated for 1 h with horseradish peroxidase conjugate of goat anti-rabbit or anti-mouse second antibody, diluted in 1:5,000 phosphate buffered saline with Tween (PBST). After washing, the membrane was processed using Super Signal West Pico chemiluminescent substrate Pierce (USA), followed by exposure to Fujifilm LAS3000 Imager Fuji (Japan). Densitometric analysis was performed with Image J densitometer using the software Excel.

Co-immunoprecipitation

BEL-7402 cells (10,000 g) were washed twice with ice-cold PBS, lysed in 1 ml of RIPA buffer for 30 min on ice, clarified by centrifugation at 4°C, and then the supernatant was subjected to immunoblot or immunoprecipitation. Each cell lysate (500 μ g) was incubated with 2 μ g appropriate antibody anti-c-Src or anti-Furin overnight at 4°C. 50 μ l of protein G was added and mixed at 4°C for 2 h with gentle agitation. The pellet was washed three times with RIPA buffer, boiled with 50 μ l 2 \times loading buffer Tris with pH 6.8, 0.1% SDS, 10% glycerol, 0.025% Bromophenol blue, 20 mM 1,4-dithiothreitol (DTT) for 5 min prior to gel loading. Proteins were detected by Western blot with anti-Furin, c-Src, MT1-MMP and VEGF-C. Some experiments substituted the secondary antibody with Clean-Blot IP Detection Reagent for clear IP/Western blot results.

Gelatin zymography

Levels of the active and latent forms of MMP-2, MMP-9 were analyzed by gelatin zymography as described in kit. BEL-7402 cells were washed with ice-cold PBS and lysed with RIPA buffer for 30 min on ice. Mixtures (12,000 g) were centrifuged at 4°C for 20 min. The supernatant was aliquoted and protein content was determined using BCA Protein Assay Reagent Pierce. After electrophoresis and washing, the gel was incubated at 37°C for 24 h, stained with Coomassie brilliant blue R250 and destained.

Wound healing assay

BEL-7402 cells were plated into 24-well plates and grown to confluence. The monolayer was artificially wounded using the tip of a sterile 200 μ l pipette. Cell debris was removed by washing with PBS. The cells were then incubated with c-Src inhibitors (10 μ M PP2 or SU6656) at the appropriate time. The cells migrated into the wounded areas were photographed. Wound closure was photographed at the indicated times in the same spot with an

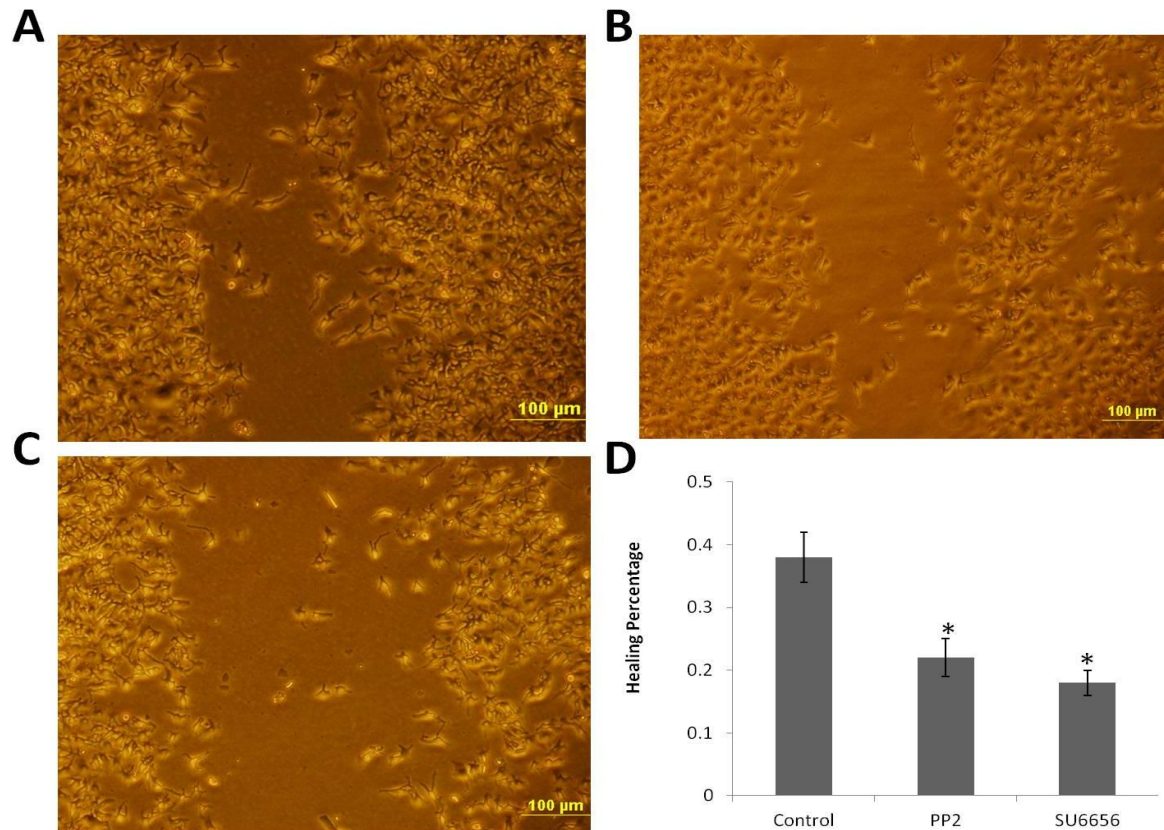


Figure 1. Effect of c-Src inhibitor on the BEL-7402 migration. Confluent 90% BEL-7402 were wounded by 200 μ l sterile pipette and then treated with c-Src inhibitors for 48 h. The cells migrated into the wounded areas were photographed. Wound closure was photographed at the indicated times at the same spot with an inverted microscope equipped with a digital camera. The extent of healing was defined as the ratio of the difference between the original and the remaining wound areas compared with the original wound area. **A.** Control group; **B.** PP2 treatment for 48 h; **C.** SU6656 treatment for 48 h; **D.** The graph represents the mean \pm S.E. of at least three independent experiments.

inverted microscope equipped with a digital camera. The extent of healing was defined as the ratio of the difference between the original and the remaining wound areas compared with the original wound area.

Transwell invasion assay

Matrigel Invasion Chambers were hydrated for 4 h before starting the invasion assay. Log-phase cells (4×10^4) were plated in 200 μ l completed RPMI 1640 containing 10% FBS in the upper chamber of the transwell, and the lower chamber was filled with 500 μ l completed RPMI 1640 containing 10% FBS. After incubation for 2 h, the cells were treated with PP2 or SU6656 as previously described for 24 h. The cells were allowed to migrate for 10 h at 37°C and 5% CO₂, followed by carrying out the invasion assay. The cells were fixed for 15 min at room temperature by replacing the culture medium in the bottom and top of the chamber with 4% formaldehyde dissolved in PBS. The cells that remained on the bottom of the chamber were stained with 0.1% crystal violet; the migrated clones were photographed under an optical microscope. The cell number was counted at 12 different areas. Data were averaged from three parallel experiments, which were normalized to that of the control.

Statistical analysis

Western blots were quantified by measuring the relative density of protein bands recognized by a particular antibody using Image J software NIH (USA). The results were expressed as mean \pm standard deviation (SD). Statistical analysis was done with Student's t-test for comparison of two groups; differences with $P < 0.05$ were considered statistically significant.

RESULTS

Effects of c-Src inhibitors on the invasion and migration of BEL-7402 cells

In order to detect whether c-Src activity regulated the invasion and migration of BEL-7402 cells, we did wound healing assay and Transwell assay. As expected, c-Src inhibitors have obvious roles in modulation of BEL-7402 cells invasion and migration. The ability of invasion and migration of the cells treated with PP2 or SU6656 decreased significantly compared with the control (Figure 1).

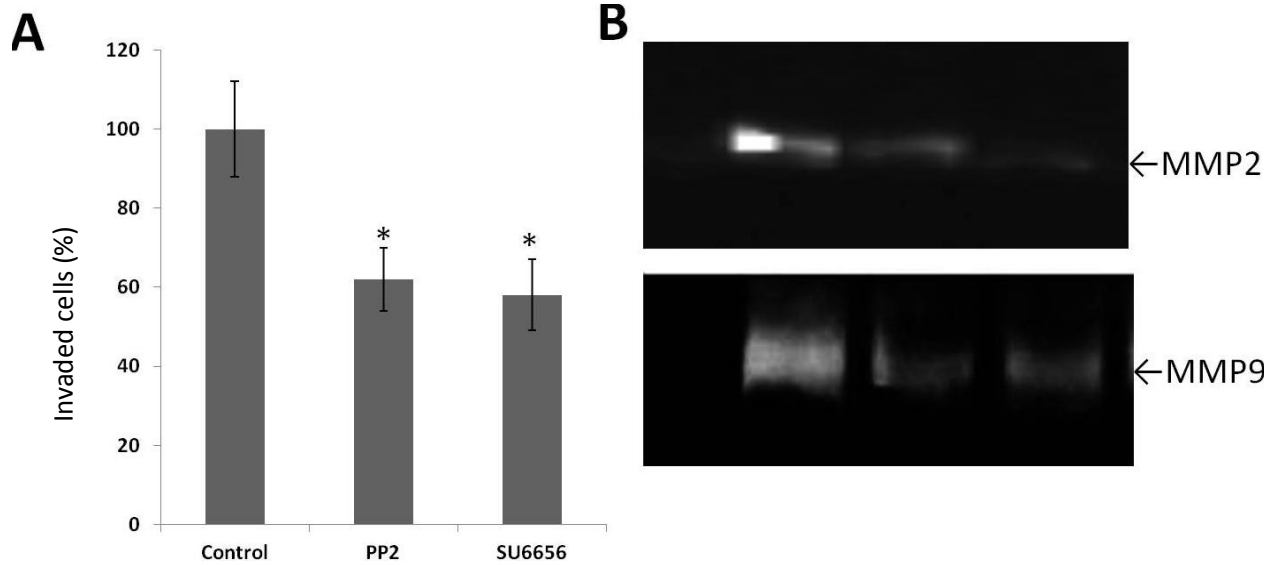


Figure 2. Effect of c-Src inhibitor on the ability of BEL-7402 cell invasion through down-regulation of the activity of MMP2 and MMP9 in BEL-7402 cells. BEL-7402 cells treated with 10 $\mu\text{mol/L}$ PP2 or SU6656 for 48 h, cell invaded ability was detected by Transwell assay. **A.** The invade cells were counted for analysis. The results shown are representative of at least three independent experiments. At the same time, the activity of MMP2 and MMP9 were detected with Zymograph assay (**B**).

Effects of c-Src inhibitors on the expression of Furin and its substrates in BEL-7402 cells

BEL-7402 cells were treated with 10 μM PP2 or SU6656 for 24 h; the expression of p-SrcY416, MT1-MMP, VEGF-C was detected by immunoblot. Upon inhibition with PP2 or SU6656, a quantitative decrease in the p-SrcY416 band intensities was observed (Figure 2A). Especially, the level of MT1-MMP, VEGF-C was also massively decreased, whereas Furin showed no obvious variation (Figure 2A). It seems that down-regulation expression of Furin substrates is in accordance with c-Src activity.

Direct binding of c-Src to Furin *in vivo*

It is not clear whether binding between c-Src and Furin may exist and how Furin interacts with its substrates in BEL-7402 cells. We then did co-immunoprecipitation of c-Src and Furin to see if c-Src is directly associated with Furin in BEL-7402 cells. As shown in Figure 3, we found that endogenous significant amounts of c-Src and Furin were specifically immunoprecipitated with the counterpart antibody for Furin and c-Src in BEL-7402 cells, respectively. The results suggest that endogenous c-Src may physically associate with Furin *in vivo*.

c-Src activity as a requirement for efficient association between Furin and its substrates

To explore the possible mechanism of the modulation of

Furin interaction with its substrates, we also analyzed the maturation of MT1-MMP or VEGF-C by Furin through co-IP experiments. Serum free BEL-7402 cells were treated as previously described. They were stimulated with 20 ng/ml PDGF-BB for 30 min or pretreated with 10 μM PP2 or SU6656; and followed by stimulation with PDGF-BB. The whole cell lysates were immunoprecipitated with anti-Furin antibody, and then detected with anti-MT1-MMP or VEGF-C antibody. Our results show that MT1-MMP can be found only in PDGF-BB stimulation group, and there was almost no band detection in SU6656 treatment group. Similar results have been found in VEGF-C detection (Figure 4). This seems that c-Src activity is necessary for efficient interaction between Furin and its substrates.

DISCUSSION

Furin has a crucial role in the progress of tumor (Cheng, 1997; Bassi et al., 2001). It may constitute a marker for tumor progression and could contribute to the prediction of the outcome of this disease (Page et al., 2007). It is a Ca^{2+} -dependent cellular endoprotease that activates a large number of precursor proteins in secretory pathway compartments (Thomas, 2002). Inhibition of Furin activity decreases substrate activation, proliferation rate and invasive potential of cancer cells, suggesting that it is a potentially useful target for therapeutics (Bassi et al., 2003).

Our present study first found that c-Src activity may directly regulate the HCC cell invasion and migration by modulating the maturation of MT1-MMP/VEGF-C. In our

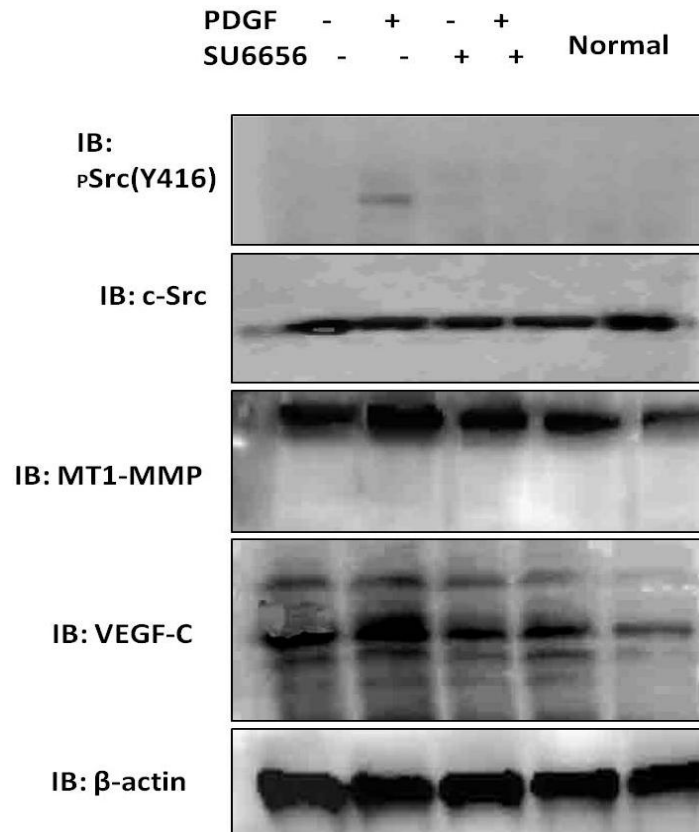


Figure 3. Expression of MT1-MMP and VEGF-C which were inhibited by c-Src inhibitor in BEL-7402 cells. BEL-7402 cells were made to undergo serum starvation for overnight, followed by incubation with 20 ng/mL PDGF-BB for 30 min. Some groups were pre-treated with SU6656 for 30 min then treated with PDGF-BB stimulation. Expression of p-c-Src, MT1-MMP and VEGF-C were detected with western blot. Protein loading control was monitored with β -actin blot.

study, we first found that the ability of BEL-7402 cells invasion and migration decreased upon PP2/SU6656 treatment. To explore the mechanism, we then detected the effects of c-Src inhibitors on the protein expression of MT1-MMP or VEGF-C in BEL-7402 cells. Results have shown that MT1-MMP or VEGF-C decreased significantly in accordance with c-Src activity, but expression of Furin had no obvious variation. The results indicate that regulation of MT1-MMP or VEGF-C does not depend on the down-regulation of Furin, another mechanism may exist.

We then hypothesized that c-Src may directly interact with Furin *in vivo* in BEL-7402 cells. Co-IP assay demonstrated that c-Src binds with Furin *in vivo*. This interaction may have a potential role in regulation of Furin activity in maturation of its substrates. So it is necessary to detect the effects of c-Src on the interaction between Furin and its substrates. MT1-MMP and VEGF-C have played a vital role in regulation of cancer cell invasion and migration. Upregulation of MT1-MMP can effectively cause elevated invasiveness in human cancer cell (Tolde

et al., 2010; Seiki, 2003; Zucker et al., 2003). To be active, the zymogen of MT1-MMP or VEGF must be cleaved to the pro-peptide by protein convertase Furin.

Our results show that activation of c-Src with PDGF-BB was found to enhance the formation of the complex between Furin and pro-MT1-MMP, but SU6656 treatment resulted in the reversion of the interaction. That is, c-Src activity is required for efficient association between Furin and its substrate pro-MT1-MMP. Similar results were seen in interaction between Furin and VEGF-C.

The platelet derived growth factor, PDGF-BB recognize both PDGFR- α and PDGFR- β subunits (Hart et al., 1988). PDGFR activation occurs through dimerization and phosphorylation of tyrosine residues in its intracellular domain, thus leading to activation of intracellular signaling, particularly of c-Src (Choudhury et al., 2006). We also found that PDGF-BB, through the activation of c-Src, regulates its own converting enzyme, Furin, creating a unique regulation loop of potential importance in a variety of cell fate and functions (Blanchette et al., 2001).

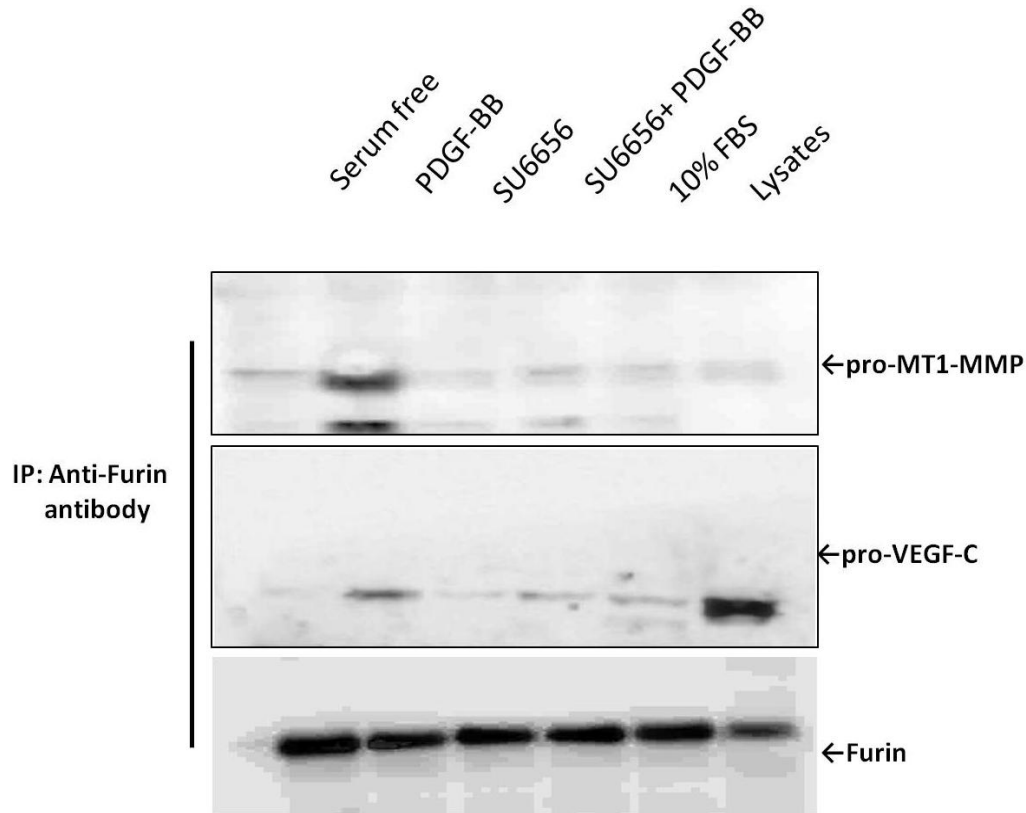


Figure 4. Modulation of furin interaction with its substrates by PDGF-BB and c-Src inhibitor in BEL-7402 cells. BEL-7402 cells were made to undergo serum starvation for overnight, then treated with PDGF-BB and SU6656 as previous description. Whole cell protein was collected and 2 μ g anti-Furin antibody was used for immunoprecipitated for overnight. The immunoprecipitates were used for detection of the expression of pre-MT1-MMP or pre-VEGF-C by specific antibody

In conclusion, we examined the role of c-Src in the process of Furin breaking down its substrates. The potential inhibitor to block Furin and subsequent processing activity are more attractive therapeutic agents for HCC cancer.

Conflict of Interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGEMENT

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Full Length Research Paper

Insecticidal activity of bioproducts on *Ceratitis capitata* Wiedemann (Diptera: Tephritidae)

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The aim of this study was to evaluate the insecticidal activity of the bioproducts progrim, essential oil from fennel and orange oil, on the mortality of *Ceratitis capitata* Wiedemann (Diptera: Tephritidae). The study was divided into three parts: the first assay studied the insecticidal activity of the products on infested fruit; secondly, a bioassay was performed by ingestion of the bioproducts in an artificial diet, and the third assay assessed the activity of the products following topical application on insects. Insecticidal activity was evaluated by application of the products at concentrations of 0.0 (control), 10,000, 15,000, 20,000, 25,000 and 30,000 ppm. In general, progrim and the essential oil of fennel induced higher mortality in larvae of *C. capitata* than orange oil. Useful information for the development of new tephritid control tools is provided by this study, because progrim and essential oil from fennel are of potential commercial importance for the control of *C. capitata*, as they cause high mortality to the larvae of *C. capitata* in infested fruit.

Key words: Bioproducts, fruit-flies, red mombin fruit, Tephritideos.

INTRODUCTION

The red mombim fruit (*Spondias purpurea* L.) is one of the most cultivated species of the genus, due to its exotic flavor and growing market acceptance. However, there is no record of a management system for the control of insect pests that damage the crop, including fruit flies. The medfly *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) attacks over 400 species of fruit, including

red mombin (Araujo et al., 2000), and is one of the main pests affecting fruit in the world, not only by direct damage to production, but also by quarantine requirements imposed by countries that import fresh fruit. Traditionally, the control of tephritids is performed by applying toxic bait with a hydrolyzed protein associated with an insecticide. Although control is effective, this

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approach leads to problems of environmental imbalance and food safety (pesticide residues in fruits) (Silva et al., 2011).

Several papers (Benelli et al., 2013; Hidayat et al., 2013; Silva et al., 2012, 2013a; Oliveira et al., 2012, 2014) have discussed the applicability of natural products such as essential oils for pest control. The insecticidal activity of essential oils may occur in several ways, causing mortality or deformation at different stages of development (Isman, 2006). Some plants produce secondary metabolites, natural or semi-synthetic precursors in the development of a chemical product, which can be used to develop new pesticides. Several substances, including phenylpropanoids and anethole in the essential oils of fennel and orange, have been shown to have insecticidal activity (Morais, 2009).

In Brazil, the search for alternatives for the management of Tephritidae is not new in the scientific environment, in view of factors that go beyond environmental contamination and health problems related to humans. Both the demand for fruit, especially without waste chemicals, and the presence of medfly has increased in developed and developing countries (Silva et al., 2011, 2013b). In order to provide information about the potential of alternative methodologies to control fruit flies, this study evaluated the insecticidal activity of the bioproducts proagrim (based on neem oil) and the essential oils from oranges and fennel.

MATERIALS AND METHODS

The insects used in this study were collected from an orchard of *Malpighia puniceifolia* L. localized in Areia, Paraíba State, Brazil. The insects were maintained in the laboratory at $25 \pm 1^\circ\text{C}$, with a 12 h photophase and relative humidity of $80 \pm 10\%$. The colonies of *C. capitata* were maintained according to the method described by Lima et al. (2008). The essential oil from orange (Prev-Am[®], sodium tetraborohydrate decahydrate) and proagrim can be freely purchased on the Brazilian market. Proagrim is a mineral compound enriched with 1% neem (Azadirachtin from *Azadirachta indica* A. Jus). The essential oil from fennel (*Foeniculum vulgare* Mill.) was obtained from seeds and fruits by hydrodistillation for 4 h with a clevenger-type apparatus. Three assays were performed with the aim of studying the insecticidal activity in red mombin fruit and to clarify the possible modes of action (ingestion or topical).

Bioassays

Insecticidal activity of the bioproducts on C. capitata in fruit

In this assay, the susceptibility of different larval instars was evaluated. Fully ripe fruits (fully yellow) were harvested, cleaned with water and 0.1% hypochlorite, and then dried at ambient temperature. For artificial infestation, 300 fruits were used, which were infested by *C. capitata* for a period of 72 h in rearing cages containing 1,500 sexually mature females. After this period, the fruits infested with eggs were placed in covered plastic containers and kept at room temperature. The fruits were sprayed with solutions (around 1500 μL of suspension/fruit) of proagrim, essential

oil of fennel or essential oil of orange at concentrations of 10,000, 15,000, 20,000, 25,000, and 30,000 ppm. The applications were performed separately in three stadiums. Larvae reached the stadiums of first, second and third instar after 24, 72, and 120 h after infestation, respectively.

Fruits without any bioproduct application were used as a control (Oliveira et al., 2012). The concentrations were chosen based on tests of these products on the physiological quality of red mombin fruit (data not shown). A group of fruits was randomly selected and maintained as a control. The sprayed fruits were re-packaged into plastic pots containing vermiculite, which were then capped and kept at room temperature under the photoperiod and humidity conditions already mentioned for larval development. The evaluation of insect mortality was performed daily after fruit infestation by counting the number of dead larvae present in the container or inside the fruits, and survival was evaluated by counting the number of pupae present in the containers. The experimental design was completely randomized with four replicates performed for each concentration/product; each replicate consisted of ten fruits. The results of this assay are expressed as mortality rate per fruit.

Application of bioproducts by ingestion

For this assay, insects were reared on an artificial diet that contained proagrim or one of the essential oils. Larvae at different developmental stages were separated into groups of 10 per dish. Each Petri dish contained 10 g of the artificial diet of carrot, yeast, and nipagin, to which was added a suspension of 1 mL of each product (proagrim, essential oil of fennel or essential oil of orange) at a concentration of 0.0 (control), 10,000, 15,000, 20,000, 25,000, or 30,000 ppm.

The products were applied to the plates with the aid of a pipette. The mortality rate of individuals was assessed daily for a period of seven days, starting from the preparation of the plates containing the insect diet products. The experimental design was completely randomized, with three replicates consisting of 10 insects for each concentration/product. Larvae were placed in Petri dishes and subjected to the same conditions of temperature, photoperiod and humidity as for the other assays.

Topical application of bioproducts

Eggs and larvae of the first, second and third instars, and pupae of *C. capitata* were used. Individuals were placed in Petri dishes and subjected to temperatures of $25 \pm 1^\circ\text{C}$, 12 h of photophase, and a relative humidity of $70 \pm 10\%$ and were provided with daily moisture according to a previously published method (Oliveira et al., 2010). Groups of larvae and pupae younger than 24 h were kept in groups of 20 per dish and were treated with proagrim or the essential oils from fennel or orange at concentrations of 0.0 (control), 10,000, 15,000, 20,000, 25,000, and 30,000 ppm (Oliveira et al., 2012). An artificial diet (10 g/dish) was provided for the larvae. Topical applications were performed with a volume of 100 μL /insect for each concentration and for each product given over the entire body of the insect. For eggs, the treatment was performed by manually spraying the whole egg chorion. Excess product that accumulated at the bottom of the Petri dish was removed with blotting paper. The mortality of individuals was evaluated daily after product application. Survival was evaluated by counting the number of hatched larvae, pupae, or adults present in the containers, to the number of applications performed on eggs, larvae, and pupae, respectively. The experimental design was completely randomized with five replicates for each concentration/product. Each replicate consisted of 20 individuals.

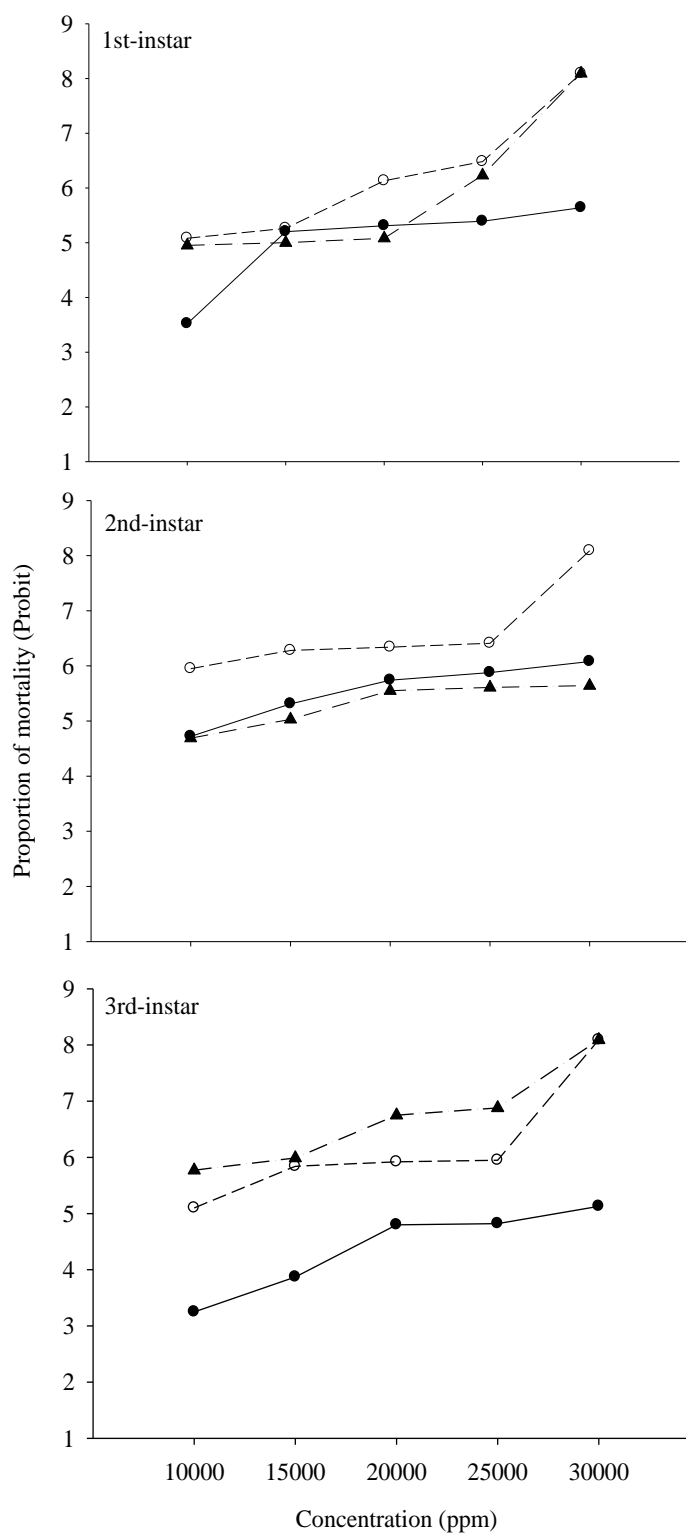


Figure 1. Proportion of mortality (Probit) of larvae of different stages of *Ceratitidis capitata* as a function of the concentration of essential oil of orange (—●—), essential oil of fennel (---○---) or proagrim (---▲---). The bioassay was conducted in artificially-infested fruits of *Spondias purpurea*.

Data analysis

The mortality data (%) of all assays were adjusted based on the mortality of the control group (Abbott, 1925) and were analyzed separately. In first assay, the results are presented as percentage mortality and were converted using the Probit scale (Finney, 1971) as was performed by Brito et al. (2009). In the first assay, the original mortality data were analyzed using the Kruskal-Wallis test ($\alpha=0.05$) (Sas, 2003). The mortalities data of the second and third assays were tested for normality (Kolmogorov normality test) (Massey, 1951) as well as homogeneity of variance (Bartlett's test) (Bartlett, 1937). The averages were compared using the Student-Newman-Keul's test ($P=0.05$) (Begun and Gabriel, 1981; Sas, 2003). The means mortalities were subjected to a mixed-model analysis of variance (three-way ANOVA) (Proc Mixed in Sas) to allow for random effects in the model. Mortality data were transformed by the arcsine of the square root to ensure homogeneity of variance and normality of the residuals. In the second assay, the 50% lethal concentrations (LC_{50}) were determined for each product and for each stadium/stage of development, and these data were subjected to Probit analysis (Finney, 1971) using the Probit procedure (Sas, 2003). A binomial model was used with a complementary log-log link function.

RESULTS

Insecticidal activity of the bioproducts on *C. capitata* in fruit

There was no difference among instars or among products at the second and third instars. However, the maximum mortality rates were of 93.00, 65.00 and 83.00% at the first instar with 30,000 ppm of fennel oil, orange oil and proagrim, respectively (Figure 1 and Table 1). There was difference among products only at a concentration of 30,000 ppm for the first instar larvae. At this concentration, the fennel oil and proagrim were more efficient than orange oil (Figure 1 and Table 1).

Application of bioproducts via ingestion

In this bioassay, there was no difference among the products ($F=1.90$; $df=2$; $P=0.1559$). However, there was a difference among instars on the mortality of fruit flies ($F=4.64$; $df=2$; $P=0.0121$). There were no interactions between product (P), concentration (C), and instar (I) ($F_{P \text{ by } C \text{ by } I(16,36)}=0.55$; $P=0.9100$).

Low levels of mortality were found in this assay. Nevertheless, comparing the susceptibility among instars of *C. capitata* larvae, applying fennel oil induced the highest mortality rates of third instar larvae to concentrations of 20,000 (10%; $P=0.012$) and 30,000 ppm (16.65%; $P=0.001$). Second instar larvae showed the greatest susceptibility to orange oil (20%; $P=0.013$) to 30,000 ppm of this oil. The greatest susceptibility of the larvae of fruit flies to proagrim was at the second instar, with mortality rates of 10% (10,000 ppm; $P<0.001$), 23.31%

Table 1. Proportion of mortality (Mean \pm SE) of *Ceratitis capitata* larvae as a function of the concentration of different products in bioassay with artificial infestation in red mombin fruits.

Concentration (ppm)	Product			χ^2	P
	Fennel oil	Orange oil	Proagrim		
1st Instar					
Control	1.23 \pm 1.11	2.22 \pm 0.98	0.98 \pm 0.45	0.2332	0.5432
10,000	50.00 \pm 28.00	60.69 \pm 3.88	37.50 \pm 23.93	0.5789	0.7487
15,000	60.41 \pm 21.34	43.75 \pm 25.74	39.26 \pm 21.99	0.2740	0.8720
20,000	65.00 \pm 23.62	53.12 \pm 27.18	48.01 \pm 20.73	0.3779	0.8728
25,000	74.10 \pm 17.70	62.14 \pm 21.92	75.00 \pm 25.00	2.1606	0.3395
30,000	92.85 \pm 7.14	65.00 \pm 21.79	83.33 \pm 9.62*	6.000	0.0498
2nd Instar					
Control	0.96 \pm 0.21	1.13 \pm 0.38	1.65 \pm 1.25	0.0980	0.9696
10000	51.11 \pm 13.22	60.28 \pm 1.38	47.55 \pm 14.63	0.5990	0.9487
15000	60.41 \pm 21.34	43.75 \pm 25.74	41.21 \pm 17.59	0.2740	0.8888
20000	62.12 \pm 23.62	63.12 \pm 22.18	58.01 \pm 20.73	0.3779	0.7723
25000	72.140 \pm 10.70	52.16 \pm 10.29	75.00 \pm 25.00	2.1606	0.4444
30000	75.23 \pm 18.70	75.00 \pm 7.70	64.12 \pm 16.20	2.2600	0.0789
3rd Instar					
Control	1.29 \pm 1.14	1.26 \pm 1.05	1.23 \pm 0.85	1.2789	0.0987
10000	64.13 \pm 22.22	69.22 \pm 1.38	64.32 \pm 12.33	1.8776	0.1076
15000	58.45 \pm 11.45	56.34 \pm 14.53	58.23 \pm 12.45	2.0080	0.2345
20000	63.11 \pm 22.52	71.14 \pm 23.23	65.09 \pm 14.65	1.4359	0.1456
25000	68.15 \pm 17.20	71.14 \pm 11.79	65.59 \pm 23.42	0.1789	0.1234
30000	69.22 \pm 16.40	69.05 \pm 12.34	65.14 \pm 12.30	0.1450	0.0898

SE= standard error. *Means are significantly different (within row) by non-parametric statistical Kruskal-Wallis test ($\alpha=0.05$).

(15,000 ppm; $P<0.001$), 10% (20,000 ppm; $P<0.001$) and 16.66% (30,000 ppm; $P<0.001$) (Table 2).

Topical application of bioproducts

Chi-square (χ^2) values were calculated to estimate the insecticidal activity of proagrim on the eggs and larvae of *C. capitata*, and the essential oil from fennel on larvae. Values were within the preset limits and, therefore, these data conformed to the Probit model. The median lethal concentration (LC₅₀) of proagrim for eggs was 17,000 ppm and was higher than that for the first, second and third instars (3,400, 2,300, and 1,800 ppm, respectively). It was not possible to estimate the LC₅₀ for proagrim treatment of pupae, due to the weak dose-response (Table 3).

There were no significant differences among the LC₅₀ values estimated for essential oil of fennel for larvae of the first, second, or third instars. It was not possible to estimate the LC₅₀ for the egg and pupal stages of *C. capitata*, due to the weak dose-response. There was no significant difference among the LC₅₀ values estimated

for proagrim and fennel oil under the studied conditions because there was an overlap in the confidence intervals (95% CI). However, for the first instar, there was no confidence interval overlap for the LC₅₀ of this product; therefore, the estimated LC₅₀ for proagrim treatment for first instar larvae of *C. capitata* was higher than that estimated for fennel oil treatment (Table 3).

No significant interactions between product (P), concentration (C) and stage (S) were observed ($F_{P \text{ by } C \text{ by } S} (32, 74) = 0.23$; $P= 1.00$). However, there was an interaction between product and stage ($F_{P \text{ by } S} (8, 74) = 0.23$; $P= 1.00$). In fact, the effect of the product varied according to fruit fly developmental stage. The results show that the mortality with proagrim treatment ranged from 35 to 59, 53 to 64, 60 to 72 and 67 to 74%, for the 10,000 to 30,000 ppm treatments for eggs, first, second and third instars, respectively (Figure 2 and Table 4).

The maximum mortality caused by the application of fennel essential oil on eggs, the first, second and third instars and pupae were 27, 77, 78, 68 and 1.80% using 30,000 ppm oil, respectively. The oil from orange resulted in lower mortality at the stadiums of larvae (L₁, L₂ and L₃) in relation to the other tested products; but, at the egg

Table 2. Mortality (Mean \pm SE) of different instars of *Ceratitis capitata* larvae as a function of the concentration of different products in bioassay following application by ingestion assay.

Concentration (ppm)	Fennel Oil				Orange Oil				Proagrim			
	1st	2nd	3rd	P>0	1st	2nd	3rd	P>0	1st	2nd	3rd	P>0
Control	0.00 \pm 0.00 ^a	0.00 \pm 0.00 ^a	0.10 \pm 0.10 ^a	0.187	1.21 \pm 0.34 ^a	2.08 \pm 0.67 ^a	1.12 \pm 0.78 ^a	0.266	0.00 \pm 0.00 ^a	0.10 \pm 0.09 ^a	0.76 \pm 0.23 ^a	0.808
10000	0.00 \pm 0.00 ^b	3.21 \pm 3.20 ^b	10.00 \pm 1.10 ^a	0.049	0.00 \pm 0.00 ^a	6.70 \pm 3.23 ^a	3.33 \pm 0.33 ^a	0.578	0.00 \pm 0.00 ^b	10.00 \pm 0.00 ^a	0.00 \pm 0.00 ^b	<0.001
15000	0.00 \pm 0.00 ^a	0.00 \pm 0.00 ^a	6.67 \pm 0.64 ^a	0.421	0.00 \pm 0.00 ^a	6.66 \pm 3.20 ^a	0.00 \pm 0.00 ^a	0.421	10.00 \pm 0.00 ^b	23.31 \pm 9.98 ^a	3.31 \pm 3.20 ^c	<0.001
20000	0.00 \pm 0.00 ^b	0.00 \pm 0.00 ^b	16.65 \pm 3.23 ^a	0.001	3.53 \pm 3.33 ^a	6.79 \pm 3.14 ^a	10.00 \pm 0.00 ^a	0.578	3.33 \pm 3.30 ^b	10.00 \pm 0.00 ^a	0.00 \pm 0.00 ^b	<0.001
25000	3.33 \pm 0.31 ^a	6.65 \pm 0.68 ^a	6.64 \pm 3.33 ^a	0.729	0.00 \pm 0.00 ^a	0.00 \pm 0.00 ^a	6.67 \pm 0.66 ^a	0.430	10.00 \pm 0.00 ^a	10.00 \pm 0.00 ^a	13.33 \pm 8.81 ^a	0.961
30000	0.00 \pm 0.00 ^b	0.00 \pm 0.00 ^b	10.00 \pm 5.76 ^a	0.012	0.00 \pm 0.00 ^b	20.00 \pm 5.27 ^a	3.23 \pm 0.34 ^b	0.013	0.00 \pm 0.00 ^c	16.66 \pm 8.81 ^a	6.87 \pm 0.65 ^b	<0.001

¹Student-Newman-Keuls test: Means followed by the same lowercase letters (within the rows and the same product) are not significantly different ($P=0.05$). SE= standard error.

Table 3. Median lethal concentrations (LC₅₀) for eggs and larvae of first, second, and third instars of *Ceratitis capitata* following topical application of proagrim and fennel oil.

Product/Stage	Slope \pm SE ¹	LC ₅₀ (95% CI) ²	χ^2	P > 0
Proagrim				
Egg	360 \pm 56	17000 (13700 - 24000) ^a	6.23	0.013
First instar	560 \pm 27	3400 (1900 - 3900) ^b	4.10	0.040
Second instar	490 \pm 24	2300 (2000 - 5500) ^b	8.12	0.044
Third instar	180 \pm 12	1800 (1200 - 2600) ^b	9.28	0.002
Pupae		nc ³		
Fennel oil				
Egg		nc ³		
First instar	500 \pm 67	5400 (4299 - 10500) ^b	5.24	0.0221
Second instar	440 \pm 47	5200 (1063 - 8500) ^b	12.96	0.0003
Third instar	470 \pm 83	5500 (1777 - 5700) ^b	4.90	0.0450
Pupae		nc ³		

Each χ^2 value refers the probability of the angular coefficient > 0; LC₅₀ = median lethal concentration; values and slopes are presented in % (w/v); values followed by the same letter are not significantly different; ¹SE= Standard Error. ²CI= confidence interval. ³nc = not calculated due to the low dose-response.

and pupal stages, there were no differences in mortality rates found after the application of this product and oil from fennel. At the egg stage, the highest mortality rates were found after topical application of proagrim. The highest mortality

rates (28.00 and 20.00%) were found after the application of 25,000 and 30,000 ppm of proagrim, respectively, at the pupal stage of fruit flies, but no significant differences were found among the products ($P > 0.05$) (Table 4).

Regardless of the fennel oil concentration, larvae of the first, second and third instars were more susceptible than eggs and pupae, because the highest mortality rates were observed at these stadiums. Moreover, at all the concentrations of

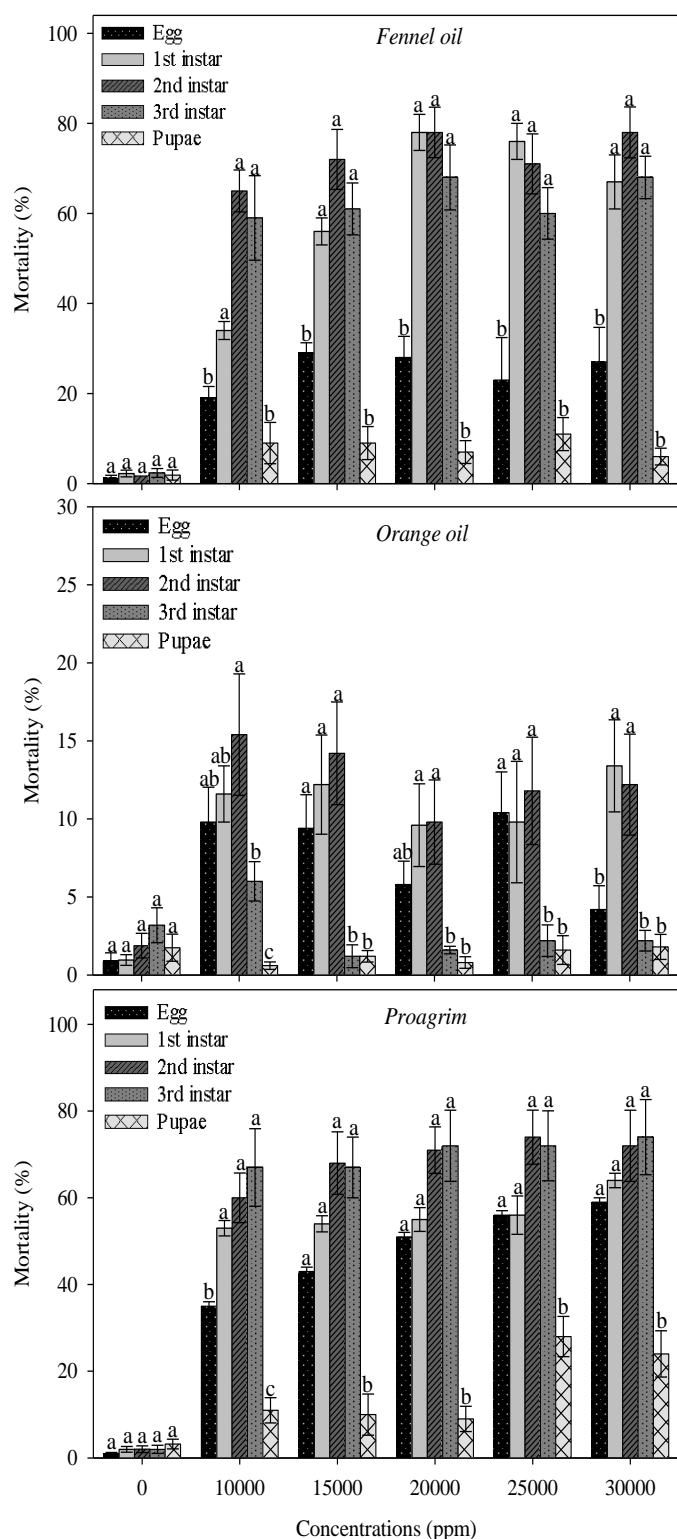


Figure 2. Mortality (%) of larvae at different stages of *Ceratitis capitata* after application of concentration of essential oil of orange, essential oil of fennel or proagrim. The bioassay was conducted by topical application. The vertical bars represent the standard deviation. Means followed by a common letter do not differ by SNK's test at 5% probability.

proagrim, the lowest mortality was found for pupae (Figure 2).

DISCUSSION

The results of this study show that the insecticidal activity of the tested products is not dependent on ingestion, as low mortality was observed when the products were applied in the diet, regardless of the larval stage evaluated. Moreover, the highest mortality of the insect was caused by proagrim or the essential oil of fennel by topical application. Mortality in the topical bioassay was influenced by the insecticide tested or the developmental stage of *C. capitata*.

Proagrim and fennel oil caused high mortality in *C. capitata* in the topical application bioassay. However, mortality was also dependent on the developmental stage of the insect. Both eggs and pupae of *C. capitata* showed lower susceptibility to fennel oil, probably because of the protection that covers the eggs and pupae, which hinders the penetration of the product. Similar results to those obtained in the present study were also found by Pinto Junior et al. (2010), who demonstrated greater susceptibility of the larvae of *Alphitobius diaperinus* Panzer (1797) (Coleoptera: Tenebrionidae) to oil of *Ocotea odorifera* (Vellozo). Evaluation of the toxic effects of neem seed cake showed that this substrate acts by contact with the larvae-pupae of *C. capitata* and leads to high mortality at concentrations higher than 50%, and also prolongs the pupal period (Silva et al., 2011). The insecticidal activity of some natural products is associated with higher concentrations, as shown for anethole (Moraes et al., 2006; Figueiredo et al., 2010). It is possible that the insecticidal action of the oil of fennel is due to this substance, since the major compound in both leaves and fruits of fennel (*F. vulgare*) is trans-anethole (Mimica-Doki et al., 2003; Sousa et al., 2005). Proagrim is a mineral compound enriched with neem (Azadirachtin). Azadirachtin acts by blocking the synthesis and release of moulting hormones (ecdysteroids) from the prothoracic gland, leading to incomplete ecdysis in immature insects. In adult female insects, a similar mechanism of action leads to sterility.

The tolerance of the eggs and pupae of *C. capitata* to the fennel oil and the pupae to proagrim might be related to the greater protection afforded to eggs by the chorion and to the rigidity of the pupae integument provided by the puparium (Oliveira et al., 2010). In eggs, the high LC_{50} found for proagrim was probably associated with the poor penetration of the product through the chorion film that covers the entire egg surface. Penetration of the insecticides might have been facilitated via alternative routes (Oliveira et al., 2011). The insecticides in this study act by contact (essential oil of fennel) and/or as a fumigant (proagrim). Pesticides and the phenylpropanoid

Table 4. Mortality (Mean \pm SE) of larvae of different stages of *Ceratitis capitata* as a function of the concentration of different products in bioassay following topical application.

Concentration (ppm)	Product			Pr > F
	Fennel oil	Orange oil	Proagrim	
Control	1.12 \pm 0.74 ^a	0.87 \pm 0.55 ^a	0.96 \pm 0.32 ^a	0.122
10000	19.68 \pm 5.56 ^b	9.88 \pm 2.23 ^b	35.09 \pm 17.02 ^a	0.187
15000	29.85 \pm 9.27 ^b	9.46 \pm 2.15 ^c	43.01 \pm 11.02 ^a	0.003
20000	28.63 \pm 14.71 ^b	5.87 \pm 1.49 ^b	51.39 \pm 7.31 ^a	0.049
25000	23.42 \pm 9.43 ^b	10.06 \pm 2.61 ^b	56.01 \pm 14.43 ^a	<0.001
30000	27.19 \pm 7.17 ^b	4.54 \pm 1.52 ^c	59.00 \pm 12.58 ^a	<0.001
1st instar				
Control	2.23 \pm 0.74 ^a	0.96 \pm 0.34 ^a	1.98 \pm 0.66 ^a	0.234
10000	69.50 \pm 9.66 ^a	11.68 \pm 1.80 ^b	53.45 \pm 11.78 ^a	0.010
15000	70.41 \pm 13.22 ^a	12.20 \pm 3.18 ^b	54.00 \pm 11.87 ^a	0.018
20000	59.23 \pm 15.60 ^a	9.60 \pm 2.65 ^b	55.42 \pm 12.74 ^a	0.005
25000	77.16 \pm 18.20 ^a	9.81 \pm 3.89 ^c	56.17 \pm 14.43 ^b	0.002
30000	77.32 \pm 19.33 ^a	13.41 \pm 2.95 ^b	64.06 \pm 11.66 ^a	0.005
2nd instar				
Control	1.68 \pm 0.44 ^a	1.88 \pm 0.79 ^a	2.03 \pm 0.77 ^a	0.559
10000	65.46 \pm 14.66 ^a	15.44 \pm 3.89 ^b	60.21 \pm 15.73 ^a	0.004
15000	72.28 \pm 9.30 ^a	14.20 \pm 3.29 ^b	68.48 \pm 17.21 ^a	0.017
20000	78.00 \pm 10.19 ^a	9.80 \pm 2.70 ^b	71.00 \pm 15.36 ^a	<0.001
25000	71.22 \pm 17.89 ^a	11.80 \pm 3.44 ^b	74.08 \pm 16.23 ^a	0.002
30000	78.00 \pm 19.53 ^a	12.20 \pm 3.23 ^b	72.16 \pm 18.20 ^a	0.0031
3rd instar				
Control	2.36 \pm 0.96 ^a	3.19 \pm 1.12 ^a	1.98 \pm 0.94 ^a	0.0987
10000	59.12 \pm 19.39 ^a	6.10 \pm 1.26 ^b	67.00 \pm 18.94 ^a	0.038
15000	61.08 \pm 15.76 ^a	1.20 \pm 0.73 ^b	67.00 \pm 17.00 ^a	0.003
20000	68.24 \pm 17.21 ^a	1.64 \pm 0.24 ^b	72.12 \pm 18.20 ^a	0.003
25000	60.20 \pm 15.73 ^a	2.20 \pm 1.01 ^b	72.14 \pm 18.06 ^a	0.002
30000	68.46 \pm 14.71 ^a	2.20 \pm 0.66 ^b	74.18 \pm 18.66 ^a	0.002
Pupae				
Control	1.88 \pm 1.12 ^a	1.75 \pm 0.87 ^a	3.22 \pm 1.12 ^a	0.1909
10000	9.00 \pm 4.58 ^a	0.63 \pm 0.24 ^a	11.29 \pm 2.91 ^a	0.082
15000	9.00 \pm 3.67 ^a	1.27 \pm 0.37 ^a	10.00 \pm 4.74 ^a	0.189
20000	7.00 \pm 2.54 ^a	0.80 \pm 0.37 ^a	9.19 \pm 2.91 ^a	0.057
25000	11.45 \pm 3.67 ^a	1.66 \pm 0.92 ^a	28.26 \pm 4.63 ^a	0.055
30000	6.33 \pm 1.87 ^a	1.88 \pm 0.80 ^a	24.22 \pm 5.33 ^a	0.058

¹Student-Newman-Keuls test: Means followed by the same lowercase letters (within the rows and the same stage/stadium) are not significantly different (P=0.05). SE= standard error.

anethole (found in the essential oil of fennel) (Silva et al., 2009) and triterpenoids and azadirachtin (in proagrim) all act by penetrating the insect body by the respiratory system. Proagrim also acts through the cuticle (Prates and Santos, 2000).

The insecticidal activity of orange oil in this study was

low for immature stages of *C. capitata*. The insecticidal activity of the essential oil from orange (sodium tetraborohydrate decahydrate) causes high mortality in the aphid *Hyadaphis foeniculi* Passerini (1860) (Hemiptera: Aphididae), ranging from 91.1 to 97.7% at concentrations of 0.3 to 0.7% (w/v), respectively (Lopes et al., 2009).

Other studies also demonstrated a potential insecticidal effect of extracts of *Citrus* species on *C. capitata* (Siskos et al., 2009).

Recent studies have shown the potential of substances from plants in the control of fruit flies (Benelli et al., 2012, 2013; Hidayat et al., 2013). In general, the LC₅₀ values for proagrim or the essential oil of fennel on *C. capitata* eggs and larvae were low in this study. In another study, the estimated LC₅₀ for adults and immature stages of *C. capitata* treated with an aqueous extract of neem kernels was 1,368 and 9,390 ppm, respectively, and 7,522 and 13,028 ppm for adults and immature adults of *Anastrepha fraterculus* (Wied.) (Diptera: Tephritidae) (Silva, 2010). The essential oils of *Rosmarinus officinalis* L. and *Salvia officinalis* L., despite being rich in hydrocarbons and ketone monoterpenes, respectively, showed low insecticidal activity, whereas the oils of *Cinnamomum zeylanicum* Blume and *Thymus* sp. exhibited significant toxic effects, leading to 90% mortality after 72 h, which could be attributed to the activity of cinnamic aldehyde (Passino, 1999).

The bioproducts in this study may be used as an integral part of integrated pest management programs, since they are of low toxicity to beneficial entomofauna (Abramson et al., 2006; Silva et al., 2009). The insecticides proagrim and essential oil from fennel can be used to control *C. capitata*, since they promoted high insect mortality at low concentrations. This study shows that proagrim and essential oil of fennel are of potential commercial importance for the control of *C. capitata*, because they cause high mortality in the larvae of *C. capitata* in infested fruit, probably by penetrating inside the fruits of red mombin.

Conclusions

The insecticidal activity of the oil from fennel was more effective for larvae of *C. capitata* than for eggs and pupae. Proagrim and the essential oil of fennel induced higher mortality in larvae of *C. capitata* compared to orange oil. The LC₅₀ values for these products indicate that only small application volumes are required.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Effect of cooling on sperm motility before and after frozen-thawed stallion semen

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The aim of this study was to assess the effect of cooling on sperm motility before and after frozen-thawed stallion semen. Fifteen ejaculates of three stallions were collected with artificial vagina. The progressive motility was determined under microscope immediately after collection, cooling (5°C for 0, 2, 7 or 24 h) before frozen-thawed and cooling (5°C for 0, 2, 7 or 24 h) after the semen was frozen-thawed. Sperm progressive motility (83.1, 78.7, 74.8 or 70.3%, respectively) was significantly different ($P < 0.05$) at different hours of cooling before freezing. Similar pattern was found when semen was subjected to cooling, frozen-thawed and cooling time resulted in a progressive reduction in motility from 39.4 to 26.9%. The motility of semen subjected only to cooling for 24 h before freezing was optimal (70.0%) for artificial insemination. Moreover, semen subjected to cooling for 7 or 24 h before and after frozen-thawed could be used still with some considerations for artificial insemination.

Key words: Stallion, semen, motility, cooling, frozen-thawed

INTRODUCTION

The best semen quality is obtained when the semen is recently collected by artificial vagina. However, for different reasons semen must be preserved during different period of times (For example, advanced age or disability to breed by a stallion, to export and/or sell semen, to inseminate estrus synchronized mares that a single stallion may not inseminate or to research, etc). Preserved semen quality is certainly a factor that impacts the rates of pregnancies in artificial insemination.

Although, a number of studies have been assayed to improve the motility and fertility of stallion semen after

cooling and/or frozen-thawed, no satisfactory results have been obtained (Martin et al., 1979; Palacios and Zarco, 1996; Rugby et al., 2001; Kavak et al., 2002; Warnke et al., 2003; Sieme et al., 2006; Clulow et al., 2008a, b; Deichsel et al., 2008). For the purpose of obtaining satisfactory results, assayed modifications in centrifugations and semen with or without a cushion fluid (Sieme et al., 2006), added substances after cryopreserved spermatozoa (Gradil and Ball, 2000) and removal of seminal plasma (Ramires et al., 2013a; b) had been studied; the incorporation of antioxidant or another

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substance to the diet of stallions (Brinsko et al., 2005; Deichsel et al., 2008). Graham and Mocé (2005) reported that the motility or another laboratory assay does not correlate well with semen fertility. Moreover efforts to keep thawed semen and employ it to freezing process have been made (McCue et al., 2004; Underwood et al., 2009; Underwood et al., 2010a,b,c). In Laboratories where frozen semen is available, this semen is sometimes thawed before examining the mare's ovaries. If follicles are not mature enough, it is required to cool the thawed semen in order to allow a greater development of the dominant follicle. In this case a deep artificial insemination (AI) is practiced using endoscopy.

The importance of this study is that it can be applied in places of sample collection where there is equipment or technical limitation for freezing semen. Therefore, a good option is to transport cooled semen (5°C) and then freeze it in a site that has the required equipment.

MATERIALS AND METHODS

Animals and location

This study was conducted at the Semen Processing Laboratory from the Veterinary Hospital "La Silla" and the Reproduction Department of the Veterinary Medicine Faculty of the Universidad Autónoma de Nuevo León.

Three jumping stallions were selected based on acceptable (>60%) total motility of spermatozoa before treatment. French Saddle or stallion 1; Oldenburg or stallion 2 and Westfalian or stallion 3 with 11, 11 and 15 years old and weighing 600, 524 and 598 kg, respectively. Horses were fed with a commercial diet with free access to water.

Semen collection

Five ejaculates from each of three stallions ($n = 3$, $r = 5$) were collected in September every 7 days by artificial vagina (Missouri, Har-Vet®), non spermicidal lubricant gel was used (H-R of Carter Products®) along with a plastic sump preheated to 37°C and a disposable paper filter (Har-Vet®). The artificial vagina was filled with water at a temperature of 52-54°C. A dummy was used for the collection, plus an ovariectomized mare previously treated with 10 mg of estradiol cypionate IM every 7 days to induce estrous and stimulate the stallion to facilitate semen collection. Before each collection, the penis was washed with warm water (37°C). Ejaculated volume was measured with a graduated cylinder then a semen drop was placed in a photometer microcuvette holder (Sperma CUE, Minitube®) to measure sperm concentration (million/ml).

Diluents preparation

Diluents Kenney E-Z mix (Animal Reproduction System) were prepared to determine sperm motility. After heat up at 37°C, portion A (water diluents) was mixed with portion B (glucose and nonfat milk solids). To avoid agglutination, semen and diluents were mixed in 1:1 proportion. Progressive motility was evaluated using ten microlitres of sperm suspension. This sample was placed on a warm glass slide and observed using phase contrast with an ocular of 10 and objective of 40 (400X).

Cooling technique

The ejaculate was divided into 4 aliquots with a concentration of 25 to 50 million sperm cells/ml. Each aliquot portion was packed in sterile plastic bags and sealed with heat, which were cooled to a temperature of 5°C by an Equitainer II (Hamilton Research, Inc. ®). Aliquots were removed at 0, 2, 7 or 24 h. Hour 0 stayed for an instant during cooling.

Freezing technique

After the cooling process, each aliquot of semen was evaluated for progressive motility. Then, aliquots were centrifuged at 400 g for 15 min. The supernatant was removed and the sperm cells were mixed with the cryopreservation medium (lactose-EDTA: Lactose solution, Glucose-EDTA, Yolk, Glycerol, Equex STM) prepared at the Semen Processing Laboratory. Straws (0.5 ml) were filled and sealed with metal pellets using a manual micropipettes sealer. A freezing cooler was used to freeze semen (Polyfoam Packers Corp®). Micropipettes were exposed in liquid nitrogen vapor for 15 min, immersed and stored in liquid nitrogen at -196°C. For thawed, straws were randomly taken from thermos and submerged in water bath (Lind berg bluem®), for one minute at 37 °C.

Re-cooling and progressive sperm motility

After freezing, straws were randomly thawed in water at 37°C, for one minute. Progressive sperm motility was evaluated at magnification 400X under a phase contrast microscope immediately after collection. The samples were maintained at 37°C and observed at 0, 5, 10 or 15 min after re-cooling (5°C) for 0, 2, 7 or 24 h after frozen-thawed semen (re-cooling hours : post-freezing minutes 0:0, 0:5, 0:10 and 0:15; 2:0, 2:5, 2:10 and 2:15 until 24 h). In all cases semen was diluted with Kenney extender to evaluate motility.

Progressive sperm motility was evaluated after cooling (5°C) for 0, 2, 7 or 24 h before freezing it and re-cooling (5°C) for 0, 2, 7 or 24 h after frozen-thawed semen.

Statistical analysis

Progressive sperm motility was evaluated after cooling (5°C) for 0, 2, 7 or 24 h before freezing it and re-cooling (5°C) for 0, 2, 7 or 24 h after frozen-thawed semen. Progressive sperm motility data were analyzed using the GENMOD procedure of SAS. The basic model included categorical variables defined above as dependent variables. The independent variable was treatment. The PDIF option was used to detect differences between treatments. The PROC MIXED procedure of SAS was used to compare motility before and after frozen-thawed stallion semen.

RESULTS

Semen volume and mean concentration ($n = 3$, $r = 5$) for stallion 1, stallion 2 and stallion 3 were 17.2, 50.2 and 77.0 ml and 427.8, 263.0 and 208.2 million/ml, respectively. The progressive sperm motility at different cooling time (0, 2, 7 or 24 h) before freezing was different ($P < 0.05$). These results showed a difference of 12.8% between hour 0 and 24 (Table 1).

As for progressive sperm motility undergoing cooling, freezing-thawing and cooling, rates were lower but with

Table 1. Progressive sperm motility (%) after different hours of cooling before freezing ($P < 0.05$).

Stallion n	Cooling Time (h)				
	0	2	7	24	
1	15	82.0	78.4	75.0	70.4
2	15	85.4	81.4	78.4	76.0
3	15	82.0	76.4	71.0	64.6
Mean		83.1 ^a	78.7 ^b	74.8 ^b	70.3 ^c

a similar behavior than those who only were cooled before semen freezing. Mean percentages ($n = 5$, $r = 3$) were (re-cooling hours: post-freezing minutes): 39.4 (0:0) and 37.4 (0:15); 39.3 (2:0) and 35.8 (2:15); 37.1 (7:0) and 33.2 (7:15); 33.7 (24:0) and 26.9 (24:15). Figure 1, shows the trends of progressive sperm motility at different hours of cooling before and after frozen-thawed.

DISCUSSION

This is the first study that stallion spermatozoa were cooled, frozen-thawed and again cooled. There are different reasons in which semen must be kept cool or frozen during different period of times. Preserved semen quality is certainly a factor that impacts the rates of pregnancies in artificial insemination (AI). In Laboratories where frozen semen is available, sometimes this semen is thawed before examining the mare's ovaries. If follicles are not mature enough, it is required to cool the thawed semen in order to allow a greater development of the dominant follicle. In this case a deep AI is practiced using endoscopy or deep AI technique.

In this study, progressive sperm motility was different at different cooling times before freezing (from 83.0 until 70.0% for 0 and 24 h, respectively). Brinsko et al. (2000), mentioned that stallion sperm quality is acceptable (for transport and use) after 24 h of cooling at 4-5°C or for up to 40 h after collection (Aurich, 2008). Moreover the results obtained herein were similar to those observed by Sieme et al. (2004), but 17.0% better than Sieme et al. (2006) who obtained 53.1% of progressive spermatozoa motility when the semen was stored at 5°C for 24 h and 30-40% better when stallion spermatozoa was cooled during 24 or 48 h (Dawson et al., 2000). There are a number of studies to improve the motility and fertility of stallion semen after cooling and/or frozen-thawed, however, no satisfactory results have been obtained (Martin et al., 1979; Palacios and Zarco, 1996; Rugby et al., 2001; Warnke et al., 2003; Sieme et al., 2006; Deichsel et al., 2008; Elhordoy et al., 2008).

To date, there are not studies regarding the use of cooled, frozen-thawed-cooled spermatozoa in domestic animals. However, there is a study in sheep where it was used for first time; the frozen-thawed, sex sorted and refrozen-thawed semen procedure with successful births

of lambs (Graaf et al., 2007). In addition, there are studies where the double frozen-thawed sperm procedure in stallion was used (McCue et al., 2004) and bull for artificial insemination (Saragusty et al., 2009; Underwood et al., 2009; 2010a; 2010b) or in vitro fertilization and production of embryos (Underwood et al., 2010c). Besides, birth of calves has been done after using the IVF and ET procedure with frozen-thawed, sex-sorted and refrozen-thawed sperm (Puglisi et al., 2006).

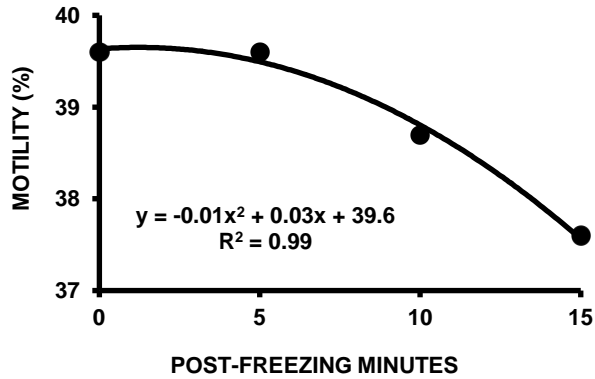
McCue et al. (2004) found that the motility of stallion spermatozoa with refreezing (double freezing) was about $45.7 \pm 10.4\%$, which was similar to that observed in our study. Besides, the same or lower results were found by Underwood et al. (2009) and Saragusty et al. (2009) who determined the in vitro progressive motility of frozen-thawed dairy bull sperm with or without sex-sorting and refreezing and thawing (0, 2 and 4 h post-thaw at 37°C; around 30.0, 10.0 and 1.0%, respectively). Moreover, when sperm of bull was frozen-thawed, sex-sorted and incubated at 15°C during 30 h the motility was around 70.0 until 12.0% (Underwood et al., 2009).

In the present study semen was made to undergo cooling, frozen-thawed and cooling procedure, the result revealed that by increasing the cooling time after being frozen-thawed reduced progressively the motility from 39.4 until 26.9%. These results were better than those reported by Warnke et al. (2003), who found after freezing and thawing stallion semen that the mean motile sperm was between 11.3 and 15.6% or 43.3 to 11.0%, when stallion spermatozoa was cold storage during 24-48 h (Dawson et al., 2000; Clulow et al., 2008b; Deichsel et al., 2008). Kavak et al. (2002) found 43.4% of spermatozoa motility when the semen was frozen-thawed. It is important to mention the large variations in semen quality from stallions and efficiency to withstand the process of freezing and thawing (Graham, 1996). However, if motility or another laboratory assay do not correlate well with semen fertility (Graham and Mocé, 2005), successful pregnancy rates using semen with 30.0% motility have been obtained (Volkman and Van Zyl, 1987).

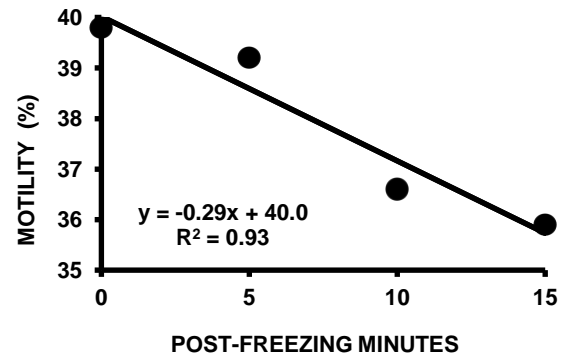
This study can be applied in places of semen collection where there is equipment or technical limitation for freezing semen. Therefore, a good option is to transport cooled semen (5 °C) and then freeze the semen in a site that has the required equipment.

Conclusion

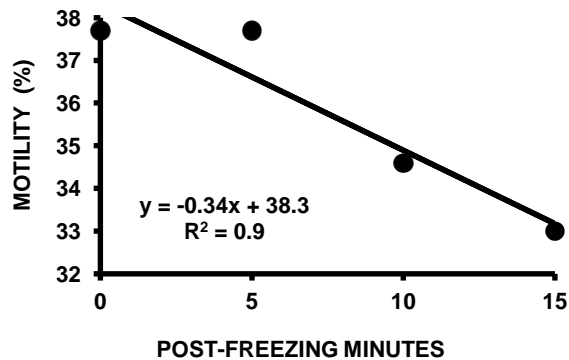
In conclusion, this study shows that stallion semen subjected only to cooling for 24 h before freezing-thawing is optimal for artificial insemination. Moreover, cooled semen before freezing-thawing and subsequent cooling or re-cooling for 7 or 24 h could be considered for AI with some limitations for example by knowing the time of ovulation and deep semen deposition in the uterine horn by deep insemination technique or endoscopy. These results indicate a good option for laboratories with limited



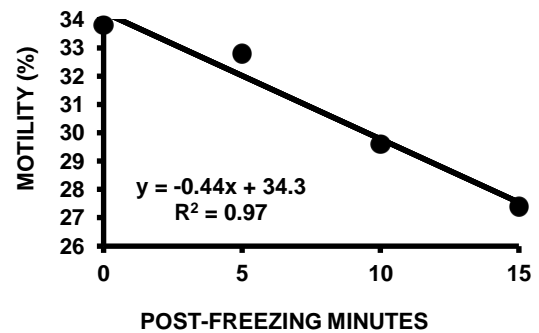
A



B



C



D

Figure 1. Sperm motility (%) in stallions ($n = 3$; $r = 5$) in function of time of cooling after frozen-thawed. Semen cooled for 0 h after thawed (A), Semen cooled for 2 h after thawed (B), Semen cooled for 7 h after thawed (C), Semen cooled for 24 h after thawed (D).

equipment or technical limitation for freezing semen. Therefore, a good option is to transport the cooled semen (5°C) and then freeze it in a laboratory with the required equipment to freeze stallion semen.


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