Volume 13 Number 41, 8 October, 2014
ISSN 1684-5315

Academic Journals

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African Journal of Biotechnology

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Vol. 13(41), pp. 4046-4051, 8 October, 2014
DOI: 10.5897/AJB2014.13903
Article Number: 522E77F47972
ISSN 1684-5315
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African Journal of Biotechnology

Full Length Research Paper

Molecular investigation of two contrasting genotypes of *Medicago truncatula* to salt stress using two expressed sequence tag-simple sequence repeat (EST-SSRs) markers

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Two expressed sequence EST-SSRs primers were used to show genetic variation and determine a potential link of these markers to salt stress tolerance on two contrasting *Medicago truncatula* genotypes (Tru 131 tolerant genotype, and *Jemalong,* sensitive one). The amplification of the DNA were isolated from 10 individual seedlings for each genotype (tolerant and sensitive) with two Expressed Sequence Tag-Simple Sequence Repeat (EST-SSR) primers (MTIC 044) and (MTIC 124) produced a total of 20 amplified products, of which MTIC 124 was polymorphic. The sizes of the alleles detected ranged from 100 to 280 bp. The EST-SSRs markers were polymorphic with an average of 1.33 alleles per primers and gave moderate values of polymorphic information content (PIC) that ranged from 0 to 0.267. The analysis of polymorphism loci for each genotype showed that the tolerant genotype (Tru 131) population had two alleles; genetic diversity index of 0.32 and PIC value of 0.267. The results obtained from unigene database of highly similarity proteins sequences with these loci showed that these two EST- SSRs loci MTIC 044 and MTIC 124 encode GATA transcription factor and cysteine proteinase inhibitor, respectively and were expressed principally in root in *M. truncatula*. This data suggest that these two loci are involved in salt stress tolerance and the two EST-SSR markers used are appropriate for the studying of salt stress tolerance in *M. truncatula*.

Key words: *Medicago truncatula*, salt stress, *in silico* analysis, expressed sequence tag-simple sequence repeat (EST-SSR), UniGene / UniProt databases.

INTRODUCTION

Medicago truncatula is widely used as a model legume plant for understanding tolerance to abiotic stress (Young and Udvardi, 2009). This legume is of great interest for sustainable agriculture and ecology. Salinity stress is an important abiotic stress which significantly affects legume growth and reduces crop production worldwide. Expressed sequence tags simple sequence repeats EST-SSRs are important sources for investigation of genetic diversity and

molecular marker development and they are useful markers for many applications in genetics and plant breeding because they show variation in the expressed part of the genome. EST-SSR primers have been reported to be less polymorphic compared with genomic SSRs in crop plants because of greater DNA sequence conservation in transcribed regions (Scott et al., 2000). The transcription factors are proteins that modulate gene expression by binding to specific cis-acting promoter elements, thus activating or repressing the transcription of target genes (Romano and Wray, 2003). Transcriptional regulation is also important for adaptation to abiotic stresses such as drought, cold, and high salinity, and for protection from biotic stresses (Shikata et al., 2004). Transcription factors are grouped into families based on the sequence of their DNA-binding domains (Luscombe and Thornton, 2002).

Our interest focuses on GATA transcription factors that are a group of DNA binding proteins broadly distributed in eukaryotes. In plants, GATA DNA motifs have been implicated in light-dependent and nitrate-dependent control of transcription (Reyes et al., 2004); they participate in nitrogen metabolism (Scazzocchio, 2000) but little information are available in relation to abiotic stress. Another interest focuses on enzymes like proteinases that are implicated in many cellular reactions involving protein degradation, such as degradation of storage proteins; their action can be inhibited by cysteine proteinase inhibitors, or cystatins superfamily. Expression of the proteinase inhibitor genes is usually limited to specific organs or to particular phases during plant growth: germination (Botella et al., 1996), early leaf senescence (Huang et al., 2001), drought (Waldron et al., 1993) or cold and salt stresses (Pernas et al., 2000; Van der Vyver et al., 2003). Information is still limited about the regulation of these inhibitors in plants and especially in the leguminous *M. truncatula* and their possible interaction with proteinases under salt stress conditions. The aim of this study was to find out if the two EST-SSR markers used (MTIC 044 and MTIC 124) encoding GATA transcriptor factors and cysteine proteinase inhibitors, respectively, are linked or no to salt stress tolerance on two contrasting *M. truncatula* genotypes (Tru 131 the tolerant genotype and *Jemalong* the sensitive one).

MATERIALS AND METHODS

Plant material

Recently harvested seeds of two contrasting genotypes of *M. truncatula* to salt stress, Tru 131 (Tolerant) provided by the institute IDGC BelAbes (Algeria) and *Jemalong* (sensitive) as reference genotype, were used in this work for molecular characterization using the two EST-SSR markers.

DNA Extraction and PCR amplification

Total genomic DNA was extracted for each genotype, from young seedling after 7 days of germination (10 seeds by genotype). DNA was isolated using a cetyl trimetrhylammonium bromide (CTAB) method adapted from Udupa et al. (1999). The two loci (EST-SSRs) located on the chromosome 3 (LG3) (Table 1), were chosen from the set of microsatellites developed by Journet et al. (2001) in *M. truncatula* (2n=16) available in GenBank EST (http://www.ncbi.nlm.nih.gov/dbEST/). Amplification of genomic DNA was done according to Udupa et al. (1999) in a PCR reactions (10 µL) containing 50 ng of template DNA, 1 × PCR Buffer, 0.2 mM dNTPs, 10 pmole of each primer and 1 unit of Taq polymerase. The amplification profile consisted of an initial period of DNA denaturation and Taq polymerase activation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s. A final extension was done at 72°C for 7 min before cooling to 4°C. PCR products were resolved on a 6% denaturing polyacrylamide gel. After electrophoresis, the DNA bands were stained with ethidium bromide and visualized by UV. For each of the defined loci, SSR allelic composition was determined for each genotype.

Polymorphism information content (PIC) values which indicate the ability to distinguish between genotypes for each primer combination for polymorphic bands was calculated with the following formula (Anderson et al., 1993): $PIC = 1 - \Sigma P^2ij$ [Pij is the frequency of the allele i revealed using the primer j]. The genetic diversity at each locus was calculated as follows: $H_i = 1 - \Sigma Pi^2$, with H_i and Pi denoting the genetic variation index and the frequency of the number of alleles at the locus, respectively (Nei, 1973). In order to find highly similarity sequences with EST SSRs, we used UniGene database (http://www.ncbi.nlm.nih.gov/UniGene/) to determine the selected proteins similarities involved in variability of salt stress tolerance and UniProt database (http://www.uniprot.org/uniprot/) to determine their principal function.

RESULTS AND DISCUSSION

Two EST- SSRs markers of *M. truncatula* (legume model) were used to test polymorphism between two contrasting genotypes to salt stress (Tru 131 the tolerant genotype and *Jemalong* the sensitive one). Results show that the MTIC 124 locus was more polymorphic (Table 1). The amplification of the DNA isolated from 10 individual seedlings for each genotype produced a total of 20 amplified products (Figures 1 and 2). The sizes of the alleles detected ranged from 100 to 280 bp. The highest number of polymorphic bands was observed with MTIC124 locus, located on chromosome 3(LG3) and at this locus, two different alleles were observed in the tolerant genotype

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Abbreviations: EST-SSR, Expressed Sequence Tag-Simple Sequence Repeat; **LG,** Linkage Group; **CTAB,** Cetyl Trimetrhylammonium Bromide; **PIC,** Polymorphism information content; **H_i,** Genetic diversity at each locus.

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Table 1. EST-SSR markers used for variability analysis of two contrasting genotypes of *M. truncatula* (Tru 131 tolerant genotype and *Jemalong* the sensitive one) to salt stress

EST SSR markers	LG	Forward (F) and reverse (R) primers (5' - 3')	Repeat motif	Annealing temperature for PCR (°C)	GenBank EST name	References
MTIC 044	3	F : CGCGCCTTCTAGTTCTCTC R : GGGGTCTCTCTTTCTTGGA	[ACC]7	55	MtBC10F10F1 MtBC *Medicago truncatula* cDNA clone MtBC10F10 T3, mRNA sequence	Journet et al. (2001) *Medicago truncatula* ESTs from endomycorrhizal roots
MTIC 124	3	F : TGTCACGAGTGTTGGAATTTT R : TGGGTTGTCAATAATGCTCA	[TG]7	55	MtBC32B02R1 MtBC *Medicago truncatula* cDNA clone MtBC32B02 T7, mRNA sequence	

LG, Linkage group.

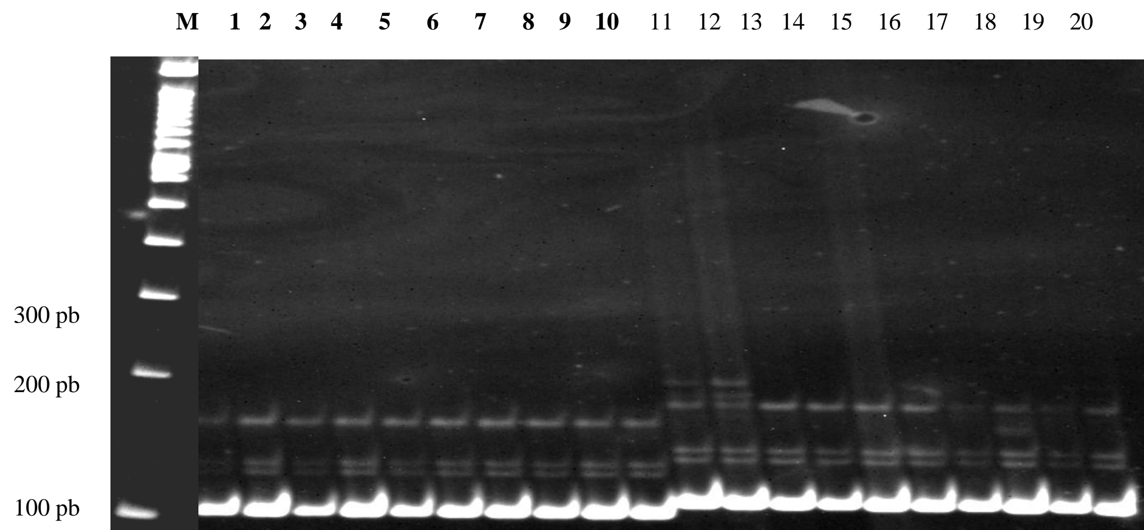

Figure 1. EST-SSR markers profile of the two contrasting genotypes of *M.truncatula* to salt stress [*Jemalong* 'sensitive': 1 to 10] and [Tru 131 ' tolerant' : 11 to 20] generated by the primer MTIC 044. M, Molecular weight marker.

(Tru 131) with genetic diversity index of 0.32 and PIC value of 0.267 (Table 2). The locus MTIC 044 located on the same chromosome 3 yielded one allele. The two EST-SSRs markers used were polymorphic with an average of 1.33 alleles per primers and gave moderate values of polymorphic information content (PIC) that ranged from 0 to 0.267. The results of EST profiles (Mtr.1896 - MTR_3g109760: GATA transcription factor and Mtr.5874 - MTR_3g043750: cysteine proteinase inhibitor) obtained from UniGene database which are of highly similarity proteins sequences to these loci showed that these two EST- SSRs loci(MTIC 044 and MTIC 124) encode GATA transcription factor and cysteine proteinase inhibitor, respectively, and were expressed principally in root in *M.truncatula* (Table 2). Their principal function was obtained from UniProt database. The EST-SSR locus

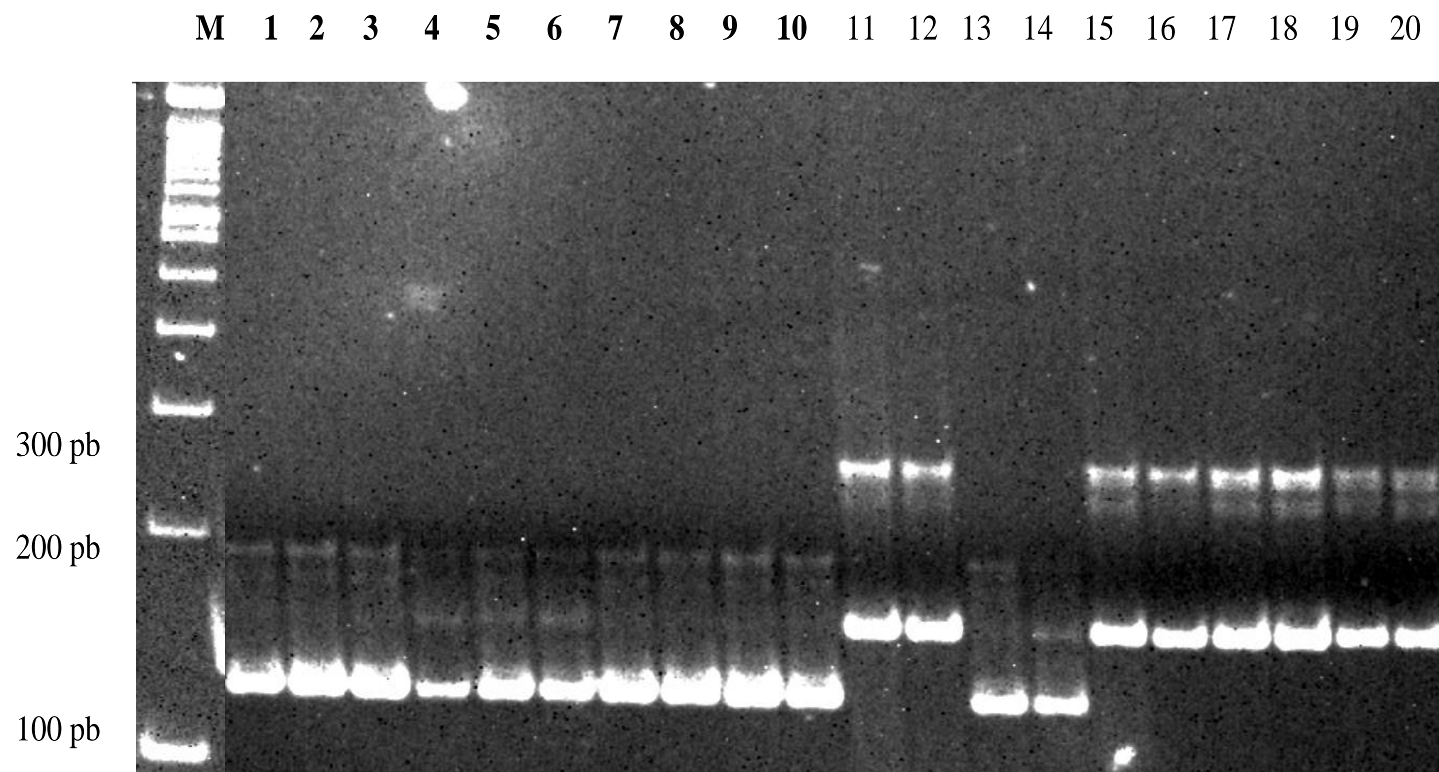

Figure 2. EST-SSR markers profile of the two contrasting genotypes of *M. truncatula* to salt stress [stress [*Jemalong* 'sensitive': 1 to 10] and [Tru 131 'tolerant' : 11 to 20] generated by the primer MTIC 124 (B). M, Molecular weight marker.

Table 2. Results of the EST-SSR markers revealed in the two contrasting genotypes of *M. truncatula* (Tru 131 tolerant and *Jemalong* sensitive one) to salt stress and data obtained from UniGene and UniProt databases of highly similarity proteins sequences with EST SSR markers used .

Genotypes	EST SSR markers	S.Z	N.A	N.G	H_i	PIC	Selected Protein Similarities	Identity %	G.A	R.E
Tru 131 (T) *Jemalong* (S)	MTIC 044	10	1	1	0	0	GATA transcription factor (MTR_3g109760) mRNA, complete cds	100	XP_003603626.1	Root
Tru 131 (T) *Jemalong* (S)	MTIC 124	10	2	2	0.32	0.26	Cysteine proteinase inhibitor (MTR_3g043750) mRNA, complete cds	100	XP_003599710.1	

T, Tolerant; S, Sensitive. S.Z, Sample size; N.A, Number of alleles; N.G. number of genotypes. G.A, Gene bank accession; R.E, restricted expression; PIC, polymorphic information content; H_i, Genetic diversity; Highly informative: (PIC > 0.50); moderately informative: (0.25 < PIC <0.50) and slightly informative: (PIC < 0.25), non informative: (PIC = 0).

(MTIC 124) was more variable than the MTIC 044 locus and this variation was observed exclusively in the tolerant genotype (Tru 131); this information suggests the direct involvement of cysteine proteinase inhibitor in seedling development under salinity, especially in root.

Cysteine proteinases play an essential role in plant growth but also, in accumulation of seed storage proteins and in the response to biotic and abiotic stresses (Grudkowska and Zagdanska, 2004). Their action can be inhibited by proteinase inhibitors induced by abiotic stress. Amouri et al. (2014) showed that the tolerant genotype (Tru 131) had a higher storage protein content and increased root growth than the sensitive one (*jemalong*) suggesting the low synthesis of the cysteine proteinsae inhibitor (cystatins) in the tolerant genotype Tru 131 compared to *Jemalong.* Interestingly, this predicted data could be confirmed at transcriptomic level. Yamaguchi-Shinozaki et al. (1992) and Koizumi et al. (1993) noted that the clones rd19 and rd21 encoding different cysteine proteinases in *Arabidopsis thaliana* were induced by water deficit and were also responsive to salt stress. Several studies suggest that plant cystatins are responsive to abiotic stresses such as drought, salt, abscisic acid and cold treatment (Gaddour et al., 2001; Van der Vyver et al., 2003; Diop et al., 2004; Massonneau et al., 2005; Christova et al., 2006), although they have also been detected in vegetative tissues, including roots and leaves (Lim et al., 1996; Pernas et al., 2000).

In *A. thaliana*, two cysteine proteinase inhibitors (cystatins) designated AtCYSa and AtCYSb, were characterized. The northern blot analyses showed that the expressions of these two cystatins gene in cells and seedlings were strongly induced by multiple abiotic stresses from high salt, drought, oxidant, and cold (Zhang et al., 2008), suggesting the same mechanism in the legume model (*M. truncatula*). However, The GATA transcription factor encoded by the EST-SSR marker (MTIC 044), was not variable, wich explains the indirect involving activation of gene expression in relation to salt stress tolerance and may be implicated in common regulation network of gene expression related to plant growth and development. Members of GATA transcription factor family that have a role in development are found throughout eukaryotes, including plants, fungi, invertebrates and vertebrates. Little information was available in plant under abiotic stress (Haenlin and Waltzer, 2004). Sugimoto et al. (2003) illustrate that the family GATA transcription factor target genes respond to stress in tobacco.

Conclusion

From all data analysis, we can propose that the two EST-SSR markers used in our study are suitable for the study of salt stress tolerance in the plant model (*M. truncatula*). The MTIC 124 locus that encode cysteine proteinase inhibitor (cystatins) is more polymorphic and implicated directly in salt tolerance than the MTIC 044 locus that encode GATA transcription factor. These two loci could be used for studying transcriptional regulation of gene expression involved in salt stress tolerance in *M. Truncatula*.

Conflict of Interests

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

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Vol. 13(41), pp. 4052-4060, 8 October, 2014
DOI: 10.5897/AJB2014.14218
Article Number: 2506B1B47973
ISSN 1684-5315
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African Journal of Biotechnology

Full Length Research Paper

Targeted parallel sequencing of the *Musa* species: Searching for an alternative model system for polyploidy studies

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Received 30 September, 2014; Accepted 3 October, 2014

Modern day genomics holds the promise of solving the complexities of basic plant sciences, and of catalyzing practical advances in plant breeding. While contiguous, "base perfect" deep sequencing is a key module of any genome project; recent advances in parallel next generation sequencing (NGS) technologies has opened up new avenues for answering biological questions in moderate to large genomes of complex polyploid species like banana. Most edible cultivated bananas belong to the Eumusa section of the Musaceae, and are diploid or triploid hybrids from their wild diploid ancestors: *Musa acuminata* (A-genome) alone or from hybridization with *Musa balbisiana* (B-genome). In this study, a second-generation parallel sequencing method was implemented to identify nucleotide variants in *Musa* spp. This strategy reduced genome complexity by enrichment with a hybridization capture library, targeting primarily exons of coding genes. The resulting marker dataset was successful in sampling broadly within the A and B genome groups and their derived hybrids. The study confirms the sequence diversity of *Musa* on a genome-wide scale even in a modest subset of *Musa* cultivars. Importantly, the experimental approach undertaken here is an efficient means of producing data for the design of high and low-density nucleotide polymorphism (single-base substitutions, small insertions and deletions or INDELs) genotyping assays applicable to a wide range of *Musa* cultivars. Thus, an excellent alternative method is reported, for characterizing associations between genotypic and phenotypic variation in *Musa* by using sequence variants as molecular markers.

Key words: Sequence capture, in-solution hybridization, nucleotide variants, polyploidy, bananas (*Musa* spp.)

INTRODUCTION

Bananas (*Musa* spp.) are very important in the diets of people and national economies in the tropics and subtropics. Global production of bananas and plantains (a type of banana) was estimated at about 140 million

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Abbreviations: NGS, Next generation sequencing, **MNPs,** multi-nucleotide polymorphisms; **INDELs,** insertions and deletions; **MQ,** mapping quality; **CDS,** coding sequence; **SNPs,** single nucleotide polymorphisms; **AFLP,** amplified fragment length polymorphism.

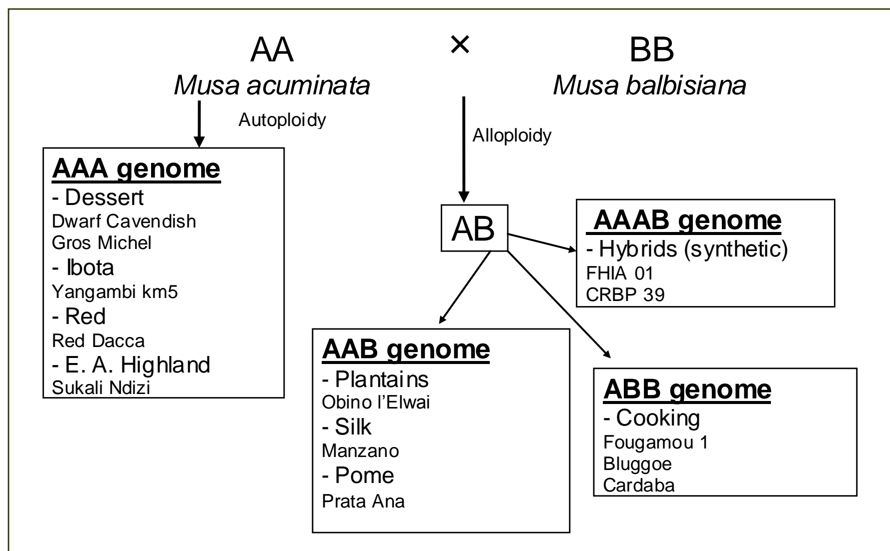

Figure 1. Schematic illustration of how a representative group of modern day edible polyploid *Musa* spp. sub-groups cultivars originated.

metric tons in 2012 (FAOSTAT, 2014). The crop is vegetatively propagated using land races and a few recently developed modern cultivars. As a consequence of its reproductive system, banana has a narrow genetic base, making it highly susceptible to diseases, pests and abiotic stresses in the environment. To genetically improve the crop, breeders depend on genetic recombination of improved diploids, crossed into seed-setting triploids to produce sterile polyploid cultivars. To facilitate modern banana improvement, breeders could benefit from an understanding of the phylogenic ancestry of the crop as well as pathways of domestication of the major cultivar groups. Markers developed to understand phylogeny and pathways of domestication could also serve in marker assisted breeding, a tool that would benefit the very difficult breeding process in *Musa.*

Most banana landraces are farmers' selection from intra- and interspecific hybrids of two diploid species, *Musa acuminata* Colla., donor of the A genome, and *Musa balbisiana* Colla., donor of the B genome (Simmonds, 1962). Most edible bananas are derived from these two diploid genomes, and are categorized into four groups (AAA, AAB, ABB and AAAB) according to the doses of the A and B genomes present (Figure 1). Natural polyploid cultivars include the triploid *M. acuminata* (AAA) sub-groups like Cavendish, Gros Michel and East African Highland banana groups, triploid hybrids (AAB) sub-groups which include the 'true' plantains, Pome and Silk types, and the triploid hybrid (ABB) cooking banana sub-groups. Synthetic tetraploid hybrids have also been produced in several genomic compositions (for example, AAAB and AABB) in international breeding programs (Escalant et al., 2002).

The East African Highland bananas (Mutika/Lujugira subgroup) belong to the AAA genome group, but are morphologically highly variable and are further classified as either beer or cooking varieties (Sebasigari, 1987; Karamura and Karamura, 1995). The AAA genome group (Figure 1) also contains the popular desert bananas, with 'Cavendish' clones and 'Gros Michel' being the most widely planted and consumed worldwide. Banana varieties with predominantly A genome (*M. acuminata*) produce sweet fruits (For example 'Giant Cavendish' - AAA genome), while those with high proportion of the B genome (*M. balbisiana*) produce starchy fruits (For example, 'Fougamou 1' - ABB genome). Tézenas du Montcel, (1988) identified seven subspecies of *M. acuminata* (namely, *microcarpa, malaccensis, burmannica, banksii, errans, burmannicoides,* and *truncata*). However, subsequent research using amplified fragment length polymorphism (AFLP) markers suggested that the seven subspecies may actually be only three subspecies: *microcarpa*, *malaccensis* and *burmannica* (Ude et al., 2002a, 2002b). Further, the subspecies *microcarpa* clustered very closely with the dessert bananas ('Gros Michel' and 'Yangambi km5'), indicating that the edible dessert bananas may have derived their A genome(s) from this taxon (Ude et al., 2002a; 2002b). Similarly, two genetic clusters were identified in *M. balbisiana* implying that the B genome is also genetically variable and that variants may be playing different roles in edible polyploid bananas (Ude et al., 2002b).

Shepherd (1988) reported that the AAB and ABB cultivars have different B genomes that arose naturally at different periods from unique parental genotypes in diverse geographical areas. However, he was not convinced that *M. balbisiana* played any role in the

Table 1. Cultivar name, identifier, group and genotype for the six *Musa* spp. germplasm accessions used in the parallel sequencing studies.

Accession name	Identifier[1]	Group[2]	Genotype
Calcutta 4	TARS 18242	subsp. *burmanicodies*	AA
Pisang Lilin	TARS 18252	subsp. *malaccensis*	AA
Tani	TARS 18046	*M. balbisiana*	BB
Manzano	TARS 17136	Silk	AAB
Obino l'Ewai	TARS 18239	Plantain	AAB
Fougamou 1	TARS 18022	Pisang Awak	ABB

[1]Germplasm maintained at the USDA-ARS Tropical Agriculture Research Station (TARS) and accessions can be found using the Germplasm Resources Information Network (GRIN). [2] Group in this case means cultivated subgroup or subspecies.

evolution of hybrid group cultivars. The AAB genome group in *Musa* consists of natural hybrids possessing 22 ‘A’ and 11 ‘B’ chromosomes. There is wide diversity in pulp characteristics and end use of the fruits of different AAB cultivars. ‘Pome’ and ‘Silk’ subgroups are the Indian and Brazil desert banana varieties that produce sub-acid, sweet fruits which are consumed fresh (Ude et al., 2002b). However, the AAB plantain subgroup produces long angular fruits with starchy pulp, which is not palatable unless cooked. Among triploids, the ABB are thought to have risen through artificial hybridization between *M. acuminata* and *M. balbisiana* followed by allopolyplodization (For example, ‘Fougamou 1’ and ‘Bluggoe’). Such subdivision within the diverse polyploids may reflect differing contributions of specific subspecies in the phylogeny of individual groups of cultivars as previously suggested (Lebot et al., 1993).

Other plants with similar genomic structure to bananas, such as *Brassica* and cotton, are considered model systems for molecular phylogeny and marker-assisted selection (Iniguez-Luy et al., 2009; Qureshi et al., 2004). With the establishment of the Global Musa Genomics Consortium (*http://www.musagenomics.org/*) in the last century and the more recent reference genome reporting for *Musa* A (D’Hont et al., 2012) and B (Davey et al., 2013) species, researchers are using advanced technologies to study the crop.

Sequence-based characterization techniques are being used to study specific groups of banana cultivars to further understand their genetic structure and their contribution to the observed diversity of morphological and phenotypic characteristics that distinguish the different polyploid sub-groups and clones.

In the present project, a parallel sequencing study targeting primarily exons of nuclear genes was conducted in a subpanel of edible *Musa* cultivars. This was carried out in an effort to strategically reduce genome complexity in this polyploid species and score for DNA sequence variants (SNPs and INDELS) in the accessible genome. The long term goal of this study is to confirm diversity sampling between A and B genomes in *Musa* over a broad range of accessions and explore whether a particular sequence variant falls within or near a gene of interest that influence any phenotypic trait. Also this study will facilitate modern banana improvement, whereby breeders could benefit from an understanding of the phylogenic ancestry of the crop as well as pathways of domestication of the major cultivar groups.

MATERIALS AND METHODS

Plant collection

The materials for this study were obtained from the USDA-ARS Tropical Agriculture Research Station, in Mayaguez, Puerto Rico. They represented the global gene pool of commercial banana, with emphasis on cultivars with high value in breeding and utilization. DNA was extracted from two accessions harboring pure *M. acuminata* AA diploid genomes (‘Calcutta 4’ representing the *burmannicoides* subsp., and ‘Pisang Lilin’ representing the *malaccensis* subsp.), one accession harboring pure *M. balbisiana* BB diploid genome (‘Tani’), two accessions representing the triploid hybrid AAB genome (‘Manzano’ from the Silk sub-group and ‘Obino l'Ewai’ from the Plantain sub-group), and one accession representing the triploid hybrid ABB genome (‘Fougamou 1’ of the Pisang Awak sub-group) (Table 1).

DNA extraction and fragmentation

Young trifoliate leaves were harvested and immediately frozen in liquid nitrogen. Frozen samples were ground with a mortar and pestle in liquid nitrogen. DNA was extracted from ~100 mg of powdered tissue using the DNeasy Plant Mini Kit (Qiagen, Alameda, CA) according to the manufacturer’s instruction (including an RNase degradation step). A NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA) was used to quantify DNA before subjecting to Solution Hybrid Selection (SHS) process. Each DNA sample was treated with NEBNext® dsDNA Fragmentase (New England Biolabs, Ipswich, MA). Fragmentation of DNA to an average size of about 300 bp was verified using Bioanalyzer High Sensitivity DNA Kits (Agilent Technologies, Santa Clara, CA).

Library preparation

The DNA samples were subjected to library preparation with insert sizes of ~300 bp for paired-end sequencing. DNA sequencing

Table 2. *Musa* spp. subset of representative genomic targets in with their chromosomal location for NimbleGen baits library development.

Locus ID	Chr.	Start	End	Function
GSMUA_Achr1P06140_001	chr1	4945027	4948846	Granule-bound starch synthase 1, chloroplastic/amyloplastic~ WAXY
GSMUA_Achr3P08340_001	chr3	5924248	5935167	Phytochrome B~ PHYB
GSMUA_Achr11P07670_001	chr11	5942667	5944644	Alcohol dehydrogenase 1~ ADH1
GSMUA_Achr6P16390_001	chr6	10918532	10920408	Floricaula/leafy homolog~ FL
GSMUA_Achr7P27540_001	chr7	28530932	28535471	Homeobox protein KNOX3~ KNOX3
GSMUA_Achr2P23000_001	chr2	21865341	21865595	MYB family transcription factor, putative, expressed~ DNAJC2
GSMUA_Achr4P00320_001	chr4	266419	267033	bZIP transcription factor domain containing protein, expressed~ bzpF
GSMUA_Achr6P29060_001	chr6	29366717	29372486	whirly transcription factor domain containing protein, expressed~ rexB
GSMUA_Achr9P06750_001	chr9	4297866	4299193	DNA-binding WRKY domain-containing protein~ WRKY71
GSMUA_Achr7P26700_001	chr7	27942477	27949823	MADS-box transcription factor 1~ MADS1
GSMUA_Achr3P15930_001	chr3	16897534	16914480	MADS-box protein CMB1~ CMB1
GSMUA_Achr2P13710_001	chr2	15734217	15735431	Putative Transcriptional regulator STERILE APETALA~ SAP
GSMUA_Achr5P28610_001	chr5	28709554	28714790	ADP-ribosylation factor GTPase-activating protein AGD7~ AGD7
GSMUA_Achr11P01660_001	chr11	1124949	1126595	RAN GTPase-activating protein 1~ RANGAP1
GSMUA_Achr10P28690_001	chr10	31422716	31424264	stress-induced protein, putative, expressed~ TIF32
GSMUA_Achr2P12750_001	chr2	15109260	15109769	Zinc finger A20 and AN1 domain-containing stress-associated protein 9~ SAP9
GSMUA_Achr8P01310_001	chr8	1107887	1108219	EARLY flowering protein, putative, expressed~ aroK
GSMUA_Achr9P08660_001	chr9	5590379	5593943	plant neutral invertase domain containing protein, expressed~ dapD
GSMUA_Achr8P22470_001	chr8	27039690	27048032	Sucrose synthase 2~ SUS2
GSMUA_Achr10P18850_001	chr10	25416529	25419440	Sucrose transport protein SUT1~ SUT1
GSMUA_Achr8P05340_001	chr8	3475343	3489014	Cellulose synthase-like protein G3~ CSLG3
GSMUA_Achr7P19410_001	chr7	22227499	22231808	Cellulose synthase A catalytic subunit 9 [UDP-forming]~ CESA9
GSMUA_Achr4P11310_001	chr4	8106521	8106880	Dehydrin Xero 1~ XERO1
GSMUA_Achr5P15820_001	chr5	12318136	12322339	Catalase isozyme 2~ CAT2
GSMUA_Achr2P05850_001	chr2	10788052	10791226	Lipoxygenase A~ LOX1.1

libraries were prepared using Kapa Library Preparation Kit reagents and protocol (Kapa Biosystems, Wilmington, MA). This was carried out with end repair of the fragmented DNA followed by A-base addition to the blunt ends of each strand, and finally adapter ligation using Illumina TruSeq Adapters, (Illumina, San Diego, CA). Each adapter had a ‘T’-base overhang on the 3’-end, providing a complementary site for ligating to the A-tailed fragmented DNA. The final recovery product was used as template in the pre-hybridization library amplification for enrichment and clean-up for subsequent steps. The library was subjected to electrophoretic evaluation in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) using a DNA 1000 chip.

Probe design

Five hundred genes distributed throughout the 11 chromosomes were targeted for enrichment, based on the most retained gene ontology categories reported for the draft *Musa* sequence (D’Hont et al., 2012). Probes included classical single copy genes (for example, *Adh1, PhyB, Lfy* and *Waxy*) and members of several transcription factor families (For example, *MYB, MADS*-box factors and *WRKY*) which were found to be over-retained after the whole-genome duplication in *Musa* α/β (banana) (D’Hont et al., 2012) (Table 2).

The length of final targets designed ranged from ~300 to 6,000 bp with a mean length of 1,500 bp and a total length of approximately ~500 kb. Hybridization probe-sets for selected targets (average size 100 bp) are designed from consensus sequences calculated from the reference ‘DH Pahang’-A genome (D’Hont et al., 2012) and by Roche NimbleGen (NimbleGen Systems, Madison, WI) using proprietary software algorithms with generalized parameters for probe sequence, hybridization temperature and length. The design of the probes for each of the 500 pairs of targets is aimed to overlap >80% of the targeted sequences (*Musa* reference genome A sequence).

Hybridization and MiSeq processing

Streptavidin-bead capture hybridization between the indexed libraries or template DNA and the biotinylated exon-derived probes was performed as previously published (Porreca et al., 2007; Gnirke et al., 2009). This was followed with post hybridization amplification (via Illumina adapters), purification of amplified samples and as before DNA quality check with Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The resulting post capture enriched libraries with 150 bp paired-end processing was subjected to sequencing on MiSeq (Illumina, San Diego, CA) performed at the Ambry Genetics facility, Aliso Viejo, CA. Normalization was conducted to ensure that an even read coverage across samples being sequenced simultaneously was achieved. For this, one lane of Illumina sequencing was performed to determine the number of sequence-able molecules per library. MiSeq data was analyzed using RTA software ver. 1.13 and corresponding read lengths is expected to be 2 × 150 bases. Data was further processed using the Picard data-processing pipeline to generate BAM files. Alignment was performed using Burrows-Wheeler Aligner (BWA) software version 0.5.9 (Durbin and Li, 2009).

Table 3. Coverage Statistics of NimbleGen probes.

Parameter	Value
Offset in bp	100
Consolidated/Padded Regions	25
Final Target Bases	127,077
Predicted Coverage	113,896
% Target Bases Covered	89.6
Target Bases Not Covered	13,181
% Target Bases Not Covered	10.4

Sequence alignment

Filtered sequence reads were aligned to the annotated *Musa* reference genome for *M. acuminata* 'Double Haploid-Pahang' [Double Haploid-Pahang is a man-made diploid or double haploid *M. acuminata* (AA) plant that was used for genome sequencing; *http://banana-genome.cirad.fr/download/musa_pseudochromosome.fna.gz*] using the BWA alignment tool, allowing a maximum of four mismatches and one gap of up to 3 bp. The Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) was then used to query reads that cannot be aligned by BWA, first against the *Musa* reference genome with an e-value cutoff of 1e22 and then against the National Center for Biotechnology Information (NCBI) database using default settings. Intron/exon boundaries were further verified using the European Molecular Biology Laboratory (EMBL) program Gene Wise [*http://www.ebi.ac.uk/Tools/psa/genewise/*] and the GBrowse tools under the Banana Genome Hub (*http://banana-genome.cirad.fr/home*). Different minimum overlap identity rate ~90% was also tested to facilitate measuring on- and off-target rates for each library and each experiment and avoid comparison of putative close paralogs.

Variant detection

Following sequencing, samples were processed using CASAVA 1.8.2 (Illumina, San Diego, CA). This processing included demultiplexing, aligning 100 bp short reads to the reference sequence obtained from the Banana Genome Hub (http://banana-genome.cirad.fr/download/musa_pseudochromosome.fna.gz), and variant calling. Variant calls were made for Qsnp scores > 20 and when coverage > 10. The variant calls from CASAVA-based pipeline were not suitable, on their own, for this analysis of multi-nucleotide polymorphisms. To resolve this, sequence variants were scored among aligned reads for covered regions, using the FreeBayes polymorphism discovery algorithm (Garrison and Gabor, 2012), restricting the calls to those originally made by the CASAVA pipeline. All samples were then combined into a single VCF file using the VCFtools program vcf-merge. Subsequently, this combined VCF file was split using VCFtools such that the coding sequence (CDS) and non-CDS regions were in separate files. The CDS regions were defined by files downloaded from the Banana Genome Hub website for *M. acuminata* 'DH-Pahang' v1. The VCF file was then searched for variants that were classified as 'TYPE=mnp' by Freebayes. Sequence variants included binary SNPs and small indels, as well as allelic series of tri-SNPs and tetra-SNPs, multi-nucleotide polymorphisms (MNPs), and INDELS with a variable number of (repetitive) nucleotides. Reads marked as duplicate, with more than seven base mismatches, more than three separate gaps, or with mapping quality (MQ) <30 were excluded for variant calling. Sequence variants adjacent to indels, which may arise due to local misalignment were filtered using Genome Analysis ToolKit or GATK (McKenna et al., 2010). Finally, each variant was annotated using snpEff (version 4.0d, with a custom-build database) to identify variants that were missense/nonsense relative to the reference sequence.

RESULTS

Sequencing and alignment

An in-solution hybridization capture library targeting primarily exons of nuclear coding genes was designed. Baits targeting genomic sequences in the enrichment library were distributed across all 11 *Musa* chromosomes (Table 2). Genomic libraries from the six *Musa* cultivars were indexed with Illumina Truseq adapters. The samples were captured in groups of three into two pools and paired-end sequenced. In total, 14,779,498 read-pairs were obtained, representing ~45 Megabases of sequencing data. The cultivar-specific sequence index could be identified in 95-98% of the read-pairs (Supplementary Table 1).

Genome space coverage

As a consequence of the enrichment method, sequences aligned not only to target regions, but also to flanking and off-target regions. After the reads were mapped to the reference sequence, there were 2,197,401 bp (~2.2 Mb) sequenced, of which 545,559 (~0.5 Mb) was in coding regions (CDS). There were 3,284 CDS regions sequenced.

Almost all genomic regions and genes targeted by the enrichment library fell within the accessible regions as shown in Table 3.

Variant detection

Variant calls were made for Qsnp scores > 20 and when coverage is > 10. The chromosome and location of the variant are listed as well as the reference and alternate alleles and the estimated genotype. Genotypes are reported as follows:

(i) HOM_REF both alleles match the reference sequence. *This will not appear in variant reports because such reports only contain variants.
(ii) HOM_ALT both alleles are the alternate allele.
(iii) HET_REF one allele is the reference and one is the alternate allele
(iv) HET_ALT both alleles are different alternate alleles

Also given is whether the variant is located in a coding region (CDS) or non-CDS. Again SNPs could be annotated relative to the reference sequence and identified as 8,831 synonymous variants, 6,631 missense variants and 221 splice variants across the six accessions.

Table 4. Overview of DNA variants observed across the six accessions in the available genome.

Variant type	Sequenced genome (2,197,401 bp)	Non-CDS[1] (1,651,842 bp)	CDS[1] (545,559 bp)
Dinucleotide SNPs	1,850	1,641	209
Trinucleotide SNPs	6	6	0
Tetranucleotide SNPs	6	4	2
Other MNPs[2]	8	8	0
INDELS	11,672	11,203	469

[1]CDS = Coding regions. [2] MNPs = Multi-nucleotide polymorphisms.

Sequence diversity analysis

Table 4 shows a total of 13,542 putative sequence variants (SNPs, MNPs and INDELS) that were identified in the accessible genome (12,862 in non-coding and 680 in the coding). The density of substitution variants (SNPs and MNPs) was 7.8 times higher in non-coding regions than in coding regions, and the INDEL density was 24 times higher in non-coding regions. Across all cultivars, an average variant density of 1/802 bp in coding regions and 1/128 bp in non-coding regions was observed.

DISCUSSION

DNA variants such as single nucleotide polymorphisms (SNPs), multi-nucleotide polymorphisms (MNPs), and insertions and deletions (INDELS) are different at the nucleotide sequence level among individuals or alleles and represent the basic units of genetic diversity (Uitdewilligen et al., 2013). Characterizing this diversity in polyploids via genotyping of sequence variants can be achieved by direct Sanger sequencing of PCR amplicons (Rickert et al., 2002; Sattarzadeh et al., 2006). However, the procedure is laborious and time-consuming, requiring unique primer sets to be designed in order to obtain uniform amplification parameters across alleles. Further, not only can the analysis not exceed a certain number of target genes, it is more expensive on a per-sequence-generated basis. Another high-throughput method to screen for polymorphic markers in different species is restriction-site-associated DNA (RAD) sequencing (Baird et al., 2008), which generates markers associated with designated genomic restriction sites. However, this method has several drawbacks. First, the nucleotide variants in the restriction site may interfere with digestion and cause null alleles in addition, the technique cannot target specific regions of interest or reduce genomic complexity.

Sequence capture is widely used for isolating targeted alleles from the background of an entire genome. The scale of the capture can range from hundreds to thousands of loci simultaneously (Metzker, 2005; Craig et al., 2008). By reducing the sequencing space per sample, this method makes multiplexing feasible, thereby reducing the overall sequencing cost. Secondly, it targets only a portion of the genome that is either necessary (for example, where a specific number of genes is required for adequate genomic coverage) or informative (for example, genes of a specific pathway) for the biological question being addressed, thereby reducing the complexity of the analysis (Grover et al, 2012). Lastly, given the redundancy of plant genomes, which typically include myriad gene duplications (Van de Peer et al., 2009; Jiao et al., 2011), and the fact that polyploidy is typical for many plants (Jiao et al., 2011), the read depth afforded by targeted NGS increases the possibility of identifying both the precise region (orthologs) of interest and its paralogs. The usual technical approaches under sequence capture involve hybridization of samples either to solid platforms/arrays or to solution-based, pooled oligonucleotide- or RNA-baits, both of which are complementary to the targeted genes (Davey et al., 2011). Probe design and synthesis may be outsourced to commercial service providers like NimbleGen and Agilent, who also provide a streamlined protocol for sequence capture suitable for laboratory use. Sequence capture is also advantageous in that *a priori* sequence information is required for only one taxon and subsequent capture baits designed to enrich the sequencing library may be used across taxa (at low levels of divergence).

Hybridization-based enrichment in plant research has increased in recent years. Fu et al. (2010) used sequential on-array hybridization to deplete repetitive elements from *Zea mays* genomic libraries, and then enrich the libraries for unique target loci. Saintenac et al. (2011) used solution hybridization to target nearly 3,500 dispersed loci (3.5 Mbp) from the larger genomes of allotetraploid wheats (*Triticum dicoccoides* and *T. durum* cv. Langdon, each nearly 10 Gb/1C) that were barcoded, pooled, and hybridized in a single reaction. Recently, Salmon et al. (2012) used a similar used a similar micro-array based hybrization capture to successfully target 500 pairs (homeologs) of selected genes in wild and domesticated *Gossypium hirsutum*, encompassing 550 kb of haploid transcript space (Salmon et al., 2012).

The recently sequenced genome of *M. acuminata* downloaded from the banana genome hub [*http://banana-*

genome.cirad.fr/download/musa_pseudochromosome.fna .gz] is appropriate for performing this next-generation parallel sequencing in banana. A solution-based sequence capture method to target *Musa* genomes for identifying native gene variation within the different subspecies of the *M. acuminata* as well as the *M. balbisiana* is reported for the first time in this work. Target enrichment allowed the achievement of sufficient sequence coverage depth across cultivars in our experiments. Importantly, bait hybridization enrichment allowed exclusion of repetitive regions of the *Musa* genome. The re-sequencing data from this method was instrumental to identify sequence variants as potential genomic markers in a non-model crop plant. We focused on single copy genes, for example from the set of conserved orthologous sequence genes (For example, *Lfy, Waxyn* and *WRKY*) and used uniquely mapped genes to define the genomic target regions.

To reduce sequencing costs, we multiplexed the different cultivars in a sequencing pool with custom index adapters. No index-specific bias was observed in the read counts and ~ 96% of the generated reads could be assigned to cultivars. We found consistent enrichment across all indexed samples, and virtually all target sequences were covered at a sufficient depth.

We used a mapping approach to align the sequencing data to the *Musa* reference genome sequence (D'Hont et al., 2012). Our observations led to detection of synonymous as well as missense variants distributed across the genome. An advantage of alignment to an annotated reference genome is that it allows prediction of whether a sequence variant falls within or near a gene of interest, and whether it is expected to cause a functional change in the protein product (For example, synonymous versus non-synonymous) that might alter the enzyme activity of the protein (Uitdewilligen et al., 2013). This can be very useful in determining whether a particular sequence variant is likely to be responsible for a phenotype of interest. A disadvantage of mapping sequence reads toward a reference sequence is that structural variation like chromosome rearrangements, inversions and large (transposon) insertions are likely to be missed. This can be partly avoided by *de novo* assembly, but computational difficulties associated with the assembly of highly diverse polyploid species like *Musa* makes mapping sequence reads to a reference sequence a more straightforward approach.

This study is the first to show high sequence diversity of *Musa* on a genome-wide scale even with a small number of cultivars. Our future endeavors aim to include a much larger number of *Musa* cultivars which will further increase the overall frequency of one variant for fewer number of bp in the cultivar population, both in non-coding and coding regions. There is a drawback with the presence of a limited draft genome and incomplete characterization for some genes. This may give rise to ambiguity in concluding whether any given gene is single copy as the result of random loss or selection. Our future efforts will also include functional characterization, phenotypic evaluations and population genetic studies of these genes as well as expanding the targeted regions further to span more intronic sequences to undermine their roles in controlling the morphological traits.

The resulting marker dataset is most useful for describing allele frequencies and nucleotide diversity. Also, our approach is an efficient means of producing data for the design of both high and low-density SNP genotyping assays applicable to a wide range of *Musa* cultivars, and the resulting tools can be used to address questions in population genetics and marker-trait association research. Thus, we report an excellent platform of using sequence variants as molecular markers which should pave the way for recognizing associations between sequence polymorphism and phenotypic variation in a polyploid species like *Musa*.

Conflict of Interests

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

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Supplementary Table 1. Cultivar-specific sequence index identified in 95-98% of the read-pairs.

Accession	Group	Read-pairs	Mb	% Perfect indices
Manzano	Silk	3491071	1047	99.16
Pisang Lilin	subsp. *malaccensis*	847170	254	95.42
Tani	*M. balbisiana*	2907131	872	99.05
Calcutta 4	subsp. *burmanicodies*	1717363	515	98.77
Obin l'Ewai	Plantain	2979951	894	98.76
Fougamou 1	Pisang Awak	2836812	851	98.66

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Vol. 13(41), pp. 4061-4069, 8 October, 2014
DOI: 10.5897/AJB2014.13719
Article Number: 6D5E23747974
ISSN 1684-5315
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African Journal of Biotechnology

Full Length Research Paper

In vitro multiple shoot bud induction and regeneration from plumule junction explants of pigeon pea [*Cajanus cajan* (L.) Mill sp.] cultivars

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Received 12 February, 2014; Accepted 29 September, 2014

The response of eleven Indian cultivars of pigeon pea for *in vitro* multiple shoot bud induction and regeneration from plumule junction explants under variable concentration of 6-benzyl amino purine (BAP), kinetin and thiadiazuron (TDZ) was assessed in the present study. The cultivar IPA-3088 showed best response with a maximum of 20 buds per explants in Murashige and Skoog (MS) media supplemented with 0.05 mgL^{-1} TDZ. Among the hormones tested, lower concentration of TDZ gave the best response for these cultivars though higher concentration of BAP was also effective in multiple shoot bud induction and regeneration from plumule junction explants. The elongation of multiple shoot buds was achieved in the same medium and the nature of the regenerants in most of the cases was shoots, though in few cases shoot buds and shoot primoridia were also observed. Rooting of plumule junction derived shootlets was found to be better in the presence of NAA as compared to IAA and IBA for most of the cultivars producing maximum number of primary roots. The best responding cultivar IPA 3088 showed efficient rooting in the presence of 0.2 mgL^{-1} of NAA. The regenerated plantlets were acclimatized in soil with percentage of acclimatization varying from 40-80% for different cultivars.

Key words: Pigeon pea, cultivars, multiple shoot bud induction, organogenesis, acclimatization, elongation, *Cajanus cajan* (L) Mill sp.

INTRODUCTION

Pigeon pea [*Cajanus cajan* (L) Mill sp.] is an important legume crop widely grown in tropical and subtropical regions of the world whose genome has been recently deciphered (Varshney et al., 2012; Singh et al., 2012). It is an important

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Abbreviations: BAP, 6-Benzyl amino purine; **TDZ,** Thiadiazuron; **NAA,** Naphthalene acetic acid; **IAA,** Indole acetic acid; **IBA,** Indole butyric acid; **MS,** Murashige and Skoog medium.

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source of protein (with 20 to 22% protein by dry weight) especially in vegetarian diets (Duke, 1981). The lack of genomic resources, and narrow genetic diversity has been a major hurdle for the breeders to develop strategies for its improvement (Saxena et al., 2010a; Bohra et al., 2011) though recently attempts have been for the development of cytoplasmic-nuclear male sterility for potential application in pigeon pea hybrid programme (Saxena et al., 2010b). Genetic transformation of pigeon pea cultivars has also been attempted (Lawrence and Koundal, 2001; Satyavathi et al., 2003; Thu et al., 2003; Prasad et al., 2004), though there is still limitation of highly efficient and reproducible *in vitro* regeneration protocol owing to its recalcitrance in tissue culture conditions. Although pigeon pea is considered to be recalcitrant crop, plant regeneration has been reported but only a few protocols could be successfully utilized for genetic transformations (Geetha et al., 1999). Recent improvements on genetic transformation (Lawrence and Koundal, 2001; Satyavathi et al., 2003; Dayal et al., 2003; Thu et al., 2003; Mohan and Krishnamurthy, 2003; Prasad et al., 2004) have demonstrated that the crop is not as recalcitrant as assumed earlier; however, limited to cultivars and some genotypes.

In vitro regeneration by organogenesis using diverse explants like leaf discs (Kumar et al., 1983; Eapen and George, 1993; George and Eapen, 1994; Eapen et al., 1998; Geetha et al., 1998; Dayal et al., 2003), cotyledons (Kumar et al., 1983; Mehta and Mohan, 1980; Kumar et al., 1984; Sarangi and Gleba, 1991; Naidu et al., 1995; Srinivasan et al., 2004; Chandra et al., 2003), cotyledonary nodes (Geetha et al., 1998; Shiva et al., 1994) and embryonal axes (George and Eapen, 1994; Sarangi and Gleba, 1991; Naidu et al., 1995; Franklin et al., 2000; Mohan and Krishnamurthy, 1998) has been reported. More than 50 diverse genotypes have been attempted for regeneration via organogenesis to develop suitable regeneration protocol amenable to transformation with desired genes (Krishna et al., 2010).

This paper reports assessment of 11 Indian cultivars of pigeon pea for *in vitro* multiple shoots bud induction and regeneration using plumule junction explants in the presence of different hormones.

MATERIALS AND METHODS

Seeds of 11 cultivars of pigeon pea namely IPA-2013, IPA-3088, Pusa-9, IPA-34, IPA-204, IPA-242, T-7, IPA-61, IPA-337, IPA-341 and IPA-98-3 were obtained from the Indian Institute of Pulses Research Kanpur, India. After repeated washes in running tap water, the seeds were surface sterilized with 1% cetrimide solution for 10 min followed by treatment with 70% ethanol for 30 s and 0.2% $HgCl_2$ for 5 min. Finally the seeds were washed 4 to 5 times with sterile double distilled water and germinated on MS medium (Murashige and Skoog, 1962) and cultures were kept under cool white fluorescent light at 25 ± 2°C. After 10 days, plumule junction explants of approximately 0.5 cm size were excised aseptically and were cultured on MS media supplemented with variable concentration of three hormones that is, BAP, kinetin, TDZ for multiple shoot bud induction and regeneration. Data about the number of buds per explants were recorded after 4 weeks of culture with two passage of sub culturing with a mean value of 10 replicates.

The MS media used in all experiments contained 3% sucrose gelled with 0.8% agar-agar (Hi-media Mumbai) and was sterilized by autoclaving after adjusting the pH to 5.8. Each sterilized culture tube (150 × 25 mm, Borosil) containing 20 ml of medium was inoculated with plumule junction explant and plugged with non-absorbent cotton (wrapped in one layer of cheese cloth), incubated under light-dark (16 to 8 h) at 25 ± 2°C with cool fluorescent light illumination. The explants with or without shoot initials were sub cultured repeatedly after 15 days. Numbers of shoot buds were counted after 30 days of inoculation.

For each experimental set up 10 explants were used with each concentration and experiment was repeated twice. After each successive subculture within 15 days, the well developed shoots were rooted on MS media with different concentration of NAA, IAA and IBA.

Rooted plants were removed from the medium, agar sticking to their roots was washed with tap water and transplanted into plastic cups or small pots, filled with autoclaved mixture of soil and sand (3:1) for hardening (Dayal et al., 2003). Plants were kept in high (90% or more) humidity and, initially low light intensities. The data were statistically analysed by standard ANOVA tool and treatment means were compared.

RESULTS AND DISCUSSION

The different explants namely leaf, shoot tips, nodes and embryonal axis along with plumule junction were subjected to *in-vitro* regeneration studies but based on regenerative as well as acclimatization percentage, plumule junction explants was further studied for multiple shoot bud induction and regeneration. The multiple shoot bud induction from plumule junction explants were analysed for 11 cultivars of pigeon pea by subjecting to MS media supplemented with variable concentration of BAP ranging from 0.5 to 4.0 mgL^{-1}. Substantial differences in the response of cultivars were observed with different concentration of BAP in terms of number of buds formed per explants. The response of multiple shoot bud induction among all 11 pigeon pea cultivars under different concentration of BAP is presented in Table 1. The overall response of cultivars for shoot bud induction can be summarized as IPA-3088> T-7> IPA-2013> IPA-61> IPA-337> IPA-242> IPA-34> IPA204= IPA-341> IPA-98-3> Pusa-9.

The cultivar IPA-3088 with a maximum of 7 buds per explants showed best response with 2.5 mgL^{-1} to BAP. Shoot bud induction from cotyledonary callus of pigeon pea on Blaydes medium with 2.25 mgL^{-1} BAP has been reported (Kumar et al., 1983). Similarly, 65% shoot buds from cotyledonary node explants of cultivar T-15-15 and SPMA-4 has been reported (Shiva et al., 1994; Mohan and Krishnamurthy, 1998). However, the maximum number of shoot buds regenerated per explants is not clear. A maximum of about 13-15 shoots from cotyedonary nodes were reported in MS media supplemented with 2 mgL^{-1} of BAP (Geetha et al., 1998). Overall the higher concentration of BAP comparatively showed better response for shoot bud induction among these cultivars. The present work enumerates the comparative account of shoot bud

Table 1. Effect of BAP on multiple shoot bud induction using plumule junction explants (number of shoots / explant) for eleven cultivars of pigeon pea after 4 weeks of culture with an average of 10 replicates and means with different letters differ significantly at p=0.05.

Cultivars	Concentration of BAP (mgL^{-1})							
	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0
	Number of shoots (Mean±S.D.)							
IPA-2013	$2.5±0.6^a$	$2.4±0.4^a$	$2.3±0.4^a$	$3.9±0.9^b$	$4.3±0.6^b$	$3.8±1.9^b$	$4.8±0.5^{ab}$	$4.1±0.5^b$
IPA-3088	$4.9±0.8^b$	$2.7±1.1^b$	$2.8±1.1^b$	$4.4±2.2^b$	$5.4±1.8^b$	$4.4±1.2^b$	$4.1±1.3^b$	$4.1±2.5^b$
Pusa-9	$1.7±0.4^a$	$3.0±0.0^b$	$3.5±0.5^{ab}$	$2.5±0.5^b$	$2.6±1.2^b$	$1.3±0.4^a$	$1.7±0.4^a$	$1.4±0.4^a$
IPA-34	$3.1±0.3^b$	$1.9±0.3^a$	$3.7±0.4^b$	$3.0±0.0^b$	$4.1±0.7^b$	$3.3±0.4^b$	$4.3±1.1^{ab}$	$4.2±0.4^b$
IPA-204	$2.0±0.0^a$	$2.0±0.0^a$	$2.5±0.6^a$	$2.6±0.4^a$	$3.8±0.4^b$	$4.2±0.6^{ab}$	$2.5±0.5^a$	$2.0±0.0^a$
IPA-242	$2.3±0.4^a$	$2.3±0.4^a$	$2.3±0.4^a$	$2.4±0.4^a$	$2.4±0.4^a$	$4.4±0.4^a$	$3.2±0.4^a$	$3.0±0.0^a$
T-7	$2.2±0.4^a$	$2.8±0.4^a$	$3.2±0.8^a$	$2.0±0.0^a$	$3.3±1.1^b$	$3.0±0.8^a$	$5.1±0.7^{ab}$	$3.2±0.4^a$
IPA-61	$3.1±0.9^a$	$3.2±0.9^b$	$3.0±0.0^a$	$3.6±0.4^a$	$2.3±0.4^a$	$3.0±0.6^a$	$3.5±0.8^b$	$4.6±0.4^{ab}$
IPA-337	$4.5±0.5^a$	$3.1±0.3^a$	$3.3±0.45^a$	$3.3±0.6^a$	$2.6±0.4^a$	$2.2±0.6^a$	$2.0±0.0^a$	$2.0±0.0^a$
IPA-341	$2.0±0.0^a$	$2.0±0.0^a$	$3.0±0.0^a$	$3.0±0.0^a$	$3.0±0.0^a$	$4.2±0.7^{ab}$	$3.6±0.4^b$	$2.0±0.0^a$
IPA-98-3	$1.0±0.0^a$	$2.0±0.0^a$	$2.0±0.0^a$	$3.8±0.4^a$	$2.0±0.0^a$	$2.5±0.5^a$	$2.0±0.0^a$	$2.0±0.0^a$

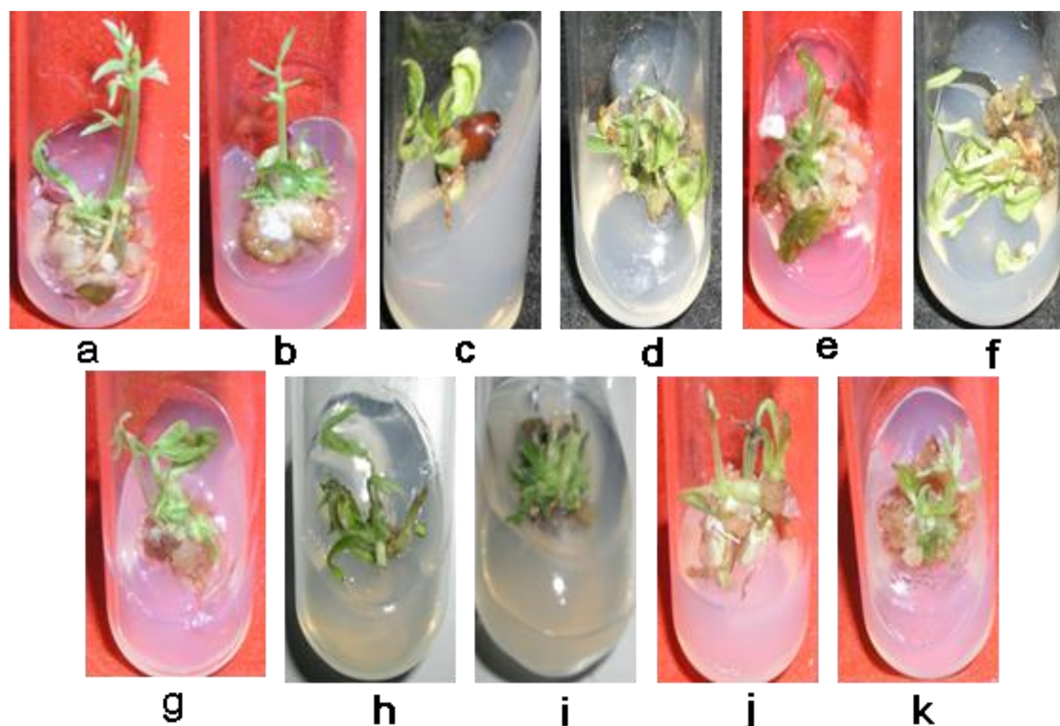

Figure 1. Multiple shoot induction from plumule junction explants of different cultivars of pigeon pea showing best response with variable concentration of BAP. **(a)** IPA2013 (3.5 mgL^{-1}); **(b)** IPA-3088 (2.5 mgL^{-1}); **(c)** Pusa-9 (1.5 mgL^{-1}); **(d)** IPA-34 (3.5 mgL^{-1}); **(e)** IPA-204 (3.0 mgL^{-1}); **(f)** IPA-242 (3.0 mgL^{-1}); **(g)** T-7 (3.5 mgL^{-1}); **(h)** IPA-61(4.0 mgL^{-1}); (i) IPA-337(0.5 mgL^{-1}); **(j)** IPA-341(3.0 mgL^{-1}); **(k)** IPA-98-3(2.0 mgL^{-1}).

induction in the case of 11 cultivars. Moreover, in this study, best responsive concentrations of BAP has been worked out and presented in Figure 1.

The cultivar IPA 3088 showed best response among all the 11 cultivars with a maximum of 10 buds per explants while only 2 buds were observed in case of IPA-61 when

Table 2. Effect of Kinetin on multiple shoot bud induction using plumule junction explants (number of shoots / explant) for eleven cultivars of pigeon pea after 4 weeks of culture with an average of 10 replicates and means with different letters differ significantly at *p*=0.05.

Cultivars	Concentration of kinetin (mgL^{-1})							
	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0
	Number of shoots (Mean±S.D.)							
IPA-2013	3.0±0.0^{a}	3.7±0.4^{a}	3.6±0.4^{a}	2.0±0.0^{a}	3.0±0.0^{a}	2.0±0.0^{a}	4.9±0.5^{a}	2.0±0.0^{a}
IPA-3088	4.2±1.2^{b}	4.1±1.3^{b}	5.4±0.8^{b}	3.6±0.4^{a}	7.5±2.1^{ab}	2.7±0.9^{a}	3.3±0.9^{a}	2.9±1.1^{a}
Pusa-9	3.5±0.5^{b}	2.0±0.0^{a}	3.5±0.5^{b}	2.0±0.0^{a}	4.1±0.7^{ab}	2.0±0.0^{a}	2.0±0.0^{a}	2.7±0.7^{b}
IPA-34	2.0±0.0^{b}	2.0±0.0^{b}	2.0±0.0^{b}	2.0±0.0^{b}	2.3±0.4^{b}	2.2±0.4^{b}	2.0±0.0^{b}	2.0±0.0^{b}
IPA-204	2.0±0.0^{a}	2.0±0.0^{a}	2.0±0.0^{a}	1.8±0.4^{a}	2.0±0.0^{a}	2.6±0.4^{b}	3.6±0.4^{ab}	2.0±0.0^{a}
IPA-242	2.0±0.0^{b}	2.0±0.0^{b}	2.0±0.0^{b}	2.0±0.0^{b}	2.0±0.0^{b}	2.8±0.8^{b}	2.0±0.0^{b}	2.0±0.0^{b}
T-7	1.0±0.0	2.0±0.0	1.0±0.0	2.0±0.0	2.0±0.0	2.0±0.0	1.0±0.0	2.0±0.0
IPA-61	1.5±0.5^{b}	1.0±0.0	2.0±0.0	1.0±0.0	1.4±0.4^{a}	1.0±0.0	1.0±0.0	1.0±0.0
IPA-337	2.0±0.0^{a}	3.5±1.3^{a}	2.0±0.0^{a}	1.0±0.0^{a}	2.0±0.0^{a}	2.0±0.0^{a}	1.0±0.0^{a}	2.0±0.0^{a}
IPA-341	2.0±0.0	2.0±0.0	1.0±0.0	2.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	2.0±0.0
IPA-98-3	1.0±0.0	2.0±0.0^{b}	2.0±0.0	1.0±0.0	2.0±0.0	1.0±0.0	1.6±0.4^{b}	1.0±0.0

subjected to MS media supplemented with variable concentration of kinetin in the range of 0.5 to 4.0 mgL^{-1}. The overall response of cultivars in terms of number of shoot buds formed per explants can be summarized as IPA-3088> IPA-2013> Pusa-9> IPA-204> IPA-337> IPA-242> IPA-334> IPA-61> T-7> IPA-341> IPA-98-3. In most of the cultivars, concentration range of 2.5 to 3.5 mgL^{-1} of kinetin was found to be most effective for shoot bud induction as shown in Table 2. In case of cultivars IPA-337 and IPA-61 lower concentration of kinetin that is, 1.0 and 1.5 mgL^{-1} was found to be effective for shoot bud induction. Regeneration using distal cotyledonary segment *via* organogenesis for diverse cultivar BDN-1, BDN-2, Gaut-82-90, ICP 7182, ICPL 87, ICPL 87119, T-15-15, TV-1 has been reported in the presence of kinetin, BAP and /or adenine sulphate (AdS) (Mohan and Krishnamurthy, 1998). The cultivar T-15-15 showed a maximum of 33 shoots in the presence of BAP + KIN + AdS. The shoot bud induction for all the eleven cultivars subjected to best responsive cocentration of kinetin is shown in Figure 2.

The same cultivar IPA-3088 responded best with 20 shoot buds per explants when cultured under TDZ at 0.05 mgL^{-1} which is quite efficient than regeneration *via* organogenesis using cotyledonary node reported for Pusa 33, ICP 8863 and ICPH cultivars with 13, 6.2 and 4.7 shoots respectively (Singh et al., 2002). The cultivar IPA-98-3 resulted in the formation of only 3 buds per explants subjected to variable concentration of TDZ. The overall response of cultivars subjected to different concentration of TDZ in terms of number of shoot buds formed per explants can be summarized as IPA 3088> IPA-61> IPA-204> T-7> IPA-34> IPA-2013> Pusa-9> IPA-341> IPA-337> IPA-242> IPA-98-3. Multiple shoot bud induction from plumule junction was comparatively better when subjected to lower concentration of TDZ, though in case of IPA-61 higher concentration that is, 0.35 mgL^{-1} was found to be better resulting in the formation of 9 buds per explants. The overall response of different concentration of TDZ for multiple shoot bud induction in all the 11 cultivars is provided in Table 3.

The shoot bud induction for all the 11 cultivars subjected to best responsive concentration of TDZ is shown in Figure 3. The percentage of explants response for multiple shooting, nature of regenerants and length of regenerated shootlets for best responsive cultivar IPA-3088 in the presence of BAP, kinetin and TDZ are presented in Table 4.

In most of the cultivars, the nature of regenerants were shoots (size more than 1 cm) though in few cases shoot buds (size less than 1 cm) were also observed. The overall response of three hormones influencing multiple shoot bud induction among all 11 cultivars clearly indicates TDZ to be better than BAP and kinetin as shown in Table-5. Further it has been observed that among the concentrations of BAP, kinetin and TDZ tried for multiple shoot bud induction, higher concentration of BAP and kinetin and lower concentration of TDZ was comparatively better for multiple shoot bud induction irrespective of cultivars.

Multiple shoot buds obtained from plumule junction explants were subjected to rooting on full strength MS basal medium supplemented with three different hormones *viz.* NAA, IAA and IBA at three different concentrations namely 0.1, 0.2 and 0.3 mgL^{-1}. NAA was found to better than other two hormones with a maximum numbers of primary roots observed by subjecting the plumule junction derived shootlets to rooting under 0.2 mgL^{-1} of NAA. The rooting response of four selected cultivars IPA-3088, IPA-Pusa-9, IPA-34 and IPA-242 is shown in Figure 4. The percentage of rooting varied from 50 to 100%. The overall response to rooting of all the 11 cultivars at three

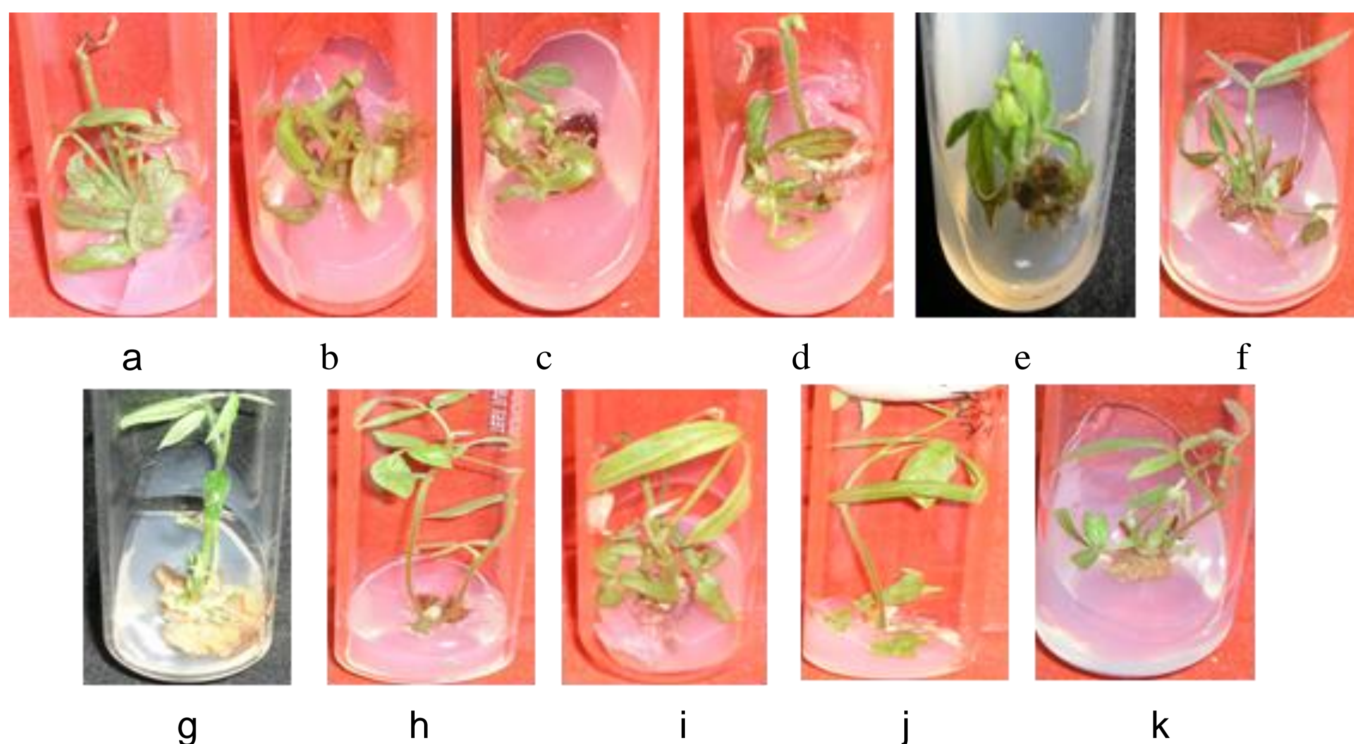

Figure 2. Multiple shoot induction from plumule junction explants of different cultivars of pigeon pea showing best response with variable concentration of kinetin **(a)** IPA2013 (3.5 mgL^{-1}); **(b)** IPA-3088 (2.5 mgL^{-1}); **(c)** Pusa-9 (2.5 mgL^{-1}); **(d)** IPA-34 (2.5 mgL^{-1}); **(e)** IPA-204 (3.5 mgL^{-1}); **(f)** IPA-242 (3.0 mgL^{-1}); **(g)** T-7 (1.0 mgL^{-1}); **(h)** IPA-61(1.5 mgL^{-1}); **(i)** IPA-337(1.0 mgL^{-1}); **(j)** IPA-341(1.0 mgL^{-1}); **(k)** IPA-98-3 (1.0 mgL^{-1}).

Table 3. Effect of TDZ on multiple shoot bud induction using plumule junction explants (number of shoots / explant) for eleven cultivars of pigeon pea after 4 weeks of culture with an average of 10 replicates and means with different letters differ significantly at p=0.05.

Cultivars	Concentration of TDZ (mgL^{-1})							
	0.05	**0.1**	**0.15**	**0.20**	**0.25**	**0.30**	**0.35**	**0.40**
	Number of shoots (Mean±S.D.)							
IPA-2013	5.0 ± 0.0^{a}	4.1 ± 0.5^{a}	3.2 ± 0.4^{a}	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
IPA-3088	12.6 ± 3.7^{ab}	10.2 ± 1.1^{b}	6.2 ± 1.7^{a}	7.9 ± 3.0^{b}	4.2 ± 1.2^{a}	10.3 ± 3.1^{b}	1.7 ± 1.1^{a}	2.2 ± 1.1^{a}
Pusa-9	3.8 ± 0.6^{b}	4.9 ± 0.7^{ab}	2.9 ± 0.8^{a}	4.1 ± 0.5^{b}	2.5 ± 0.5^{a}	3.8 ± 0.6^{b}	2.4 ± 0.4^{a}	3.6 ± 0.4^{a}
IPA-34	1.4 ± 0.4^{a}	3.5 ± 0.5^{a}	5.2 ± 0.7^{a}	3.5 ± 0.5^{a}	2.0 ± 0.0^{a}	2.0 ± 0.0^{a}	2.0 ± 0.0^{a}	1.6 ± 0.5^{a}
IPA-204	2.3 ± 0.4^{a}	4.5 ± 0.6^{a}	4.4 ± 0.4^{a}	7.7 ± 1.5^{a}	3.5 ± 0.5^{a}	3.5 ± 0.5^{a}	3.0 ± 0.0^{a}	3.0 ± 0.0^{a}
IPA-242	1.3 ± 0.4^{a}	2.0 ± 0.0^{a}	1.3 ± 0.4^{a}	2.5 ± 0.5^{b}	3.3 ± 0.4^{ab}	2.0 ± 0.0^{a}	2.0 ± 0.0^{a}	2.0 ± 0.0^{a}
T-7	2.7 ± 0.4^{a}	3.2 ± 0.4^{a}	3.4 ± 0.4^{a}	5.6 ± 0.4^{a}	3.2 ± 0.4^{a}	2.8 ± 0.4^{a}	2.6 ± 0.4^{a}	2.5 ± 0.5^{a}
IPA-61	2.0 ± 0.0^{a}	2.0 ± 0.0^{a}	2.0 ± 0.0^{a}	2.0 ± 0.0^{a}	2.0 ± 0.0^{a}	4.3 ± 0.4^{a}	7.8 ± 0.7^{a}	3.0 ± 0.0^{a}
IPA-337	3.3 ± 0.4^{b}	2.0 ± 0.0^{a}	2.8 ± 0.4^{a}	2.0 ± 0.0^{a}	4.3 ± 0.6^{ab}	2.0 ± 0.0^{a}	2.0 ± 0.0^{a}	3.1 ± 1.2^{b}
IPA-341	2.0 ± 0.0^{a}	2.0 ± 0.0^{a}	2.0 ± 0.0^{a}	2.0 ± 0.0^{a}	4.4 ± 0.4^{a}	1.6 ± 0.4^{a}	2.0 ± 0.0^{a}	2.0 ± 0.0^{a}
IPA-98-3	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0

different concentrations of NAA is represented in Table 6.

Among all the cultivars, IPA-242 showed better response to rooting, though the shootlets derived from best responsive cultivar for multiple shoot bud induction that is, IPA-3088 also showed good response in MS media supplanted with 0.2 mg/L of NAA. Similarly the shootlets derived from plumule junction explants of different cultivars were also subjected to rooting with

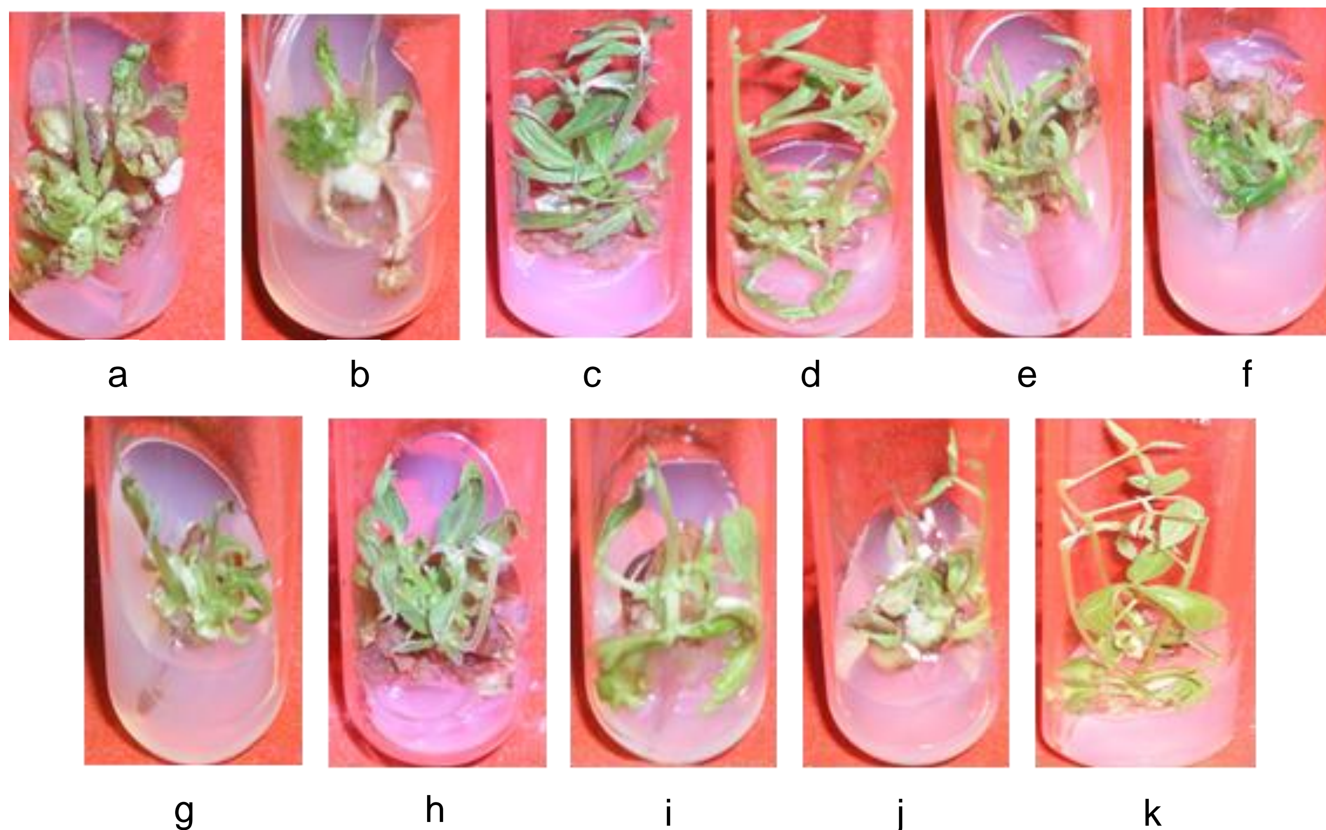

Figure 3. Multiple shoot induction from plumule junction explants of different cultivars of pigeon pea showing best response with variable concentration of TDZ (in mgL^{-1}). **(a)** IPA2013 (0.05 mgL^{-1}); **(b)** IPA-3088 (0.05 mgL^{-1}); **(c)** Pusa-9 (0.1 mgL^{-1}); **(d)** IPA-34(0.15 mgL^{-1}); **(e)** IPA-204 (0.20 mgL^{-1}); **(f)** IPA-242 (0.25 mgL^{-1}); **(g)** T-7 (0.20 mgL^{-1}); **(h)** IPA-61(0.35 mgL^{-1}); **(i)** IPA-337(0.25 mgL^{-1}); **(j)** IPA-341(0.25 mgL^{-1}); **(k)** IPA-98-3 (0.05 mgL^{-1}).

Table 4. Effect of different concentration of BAP, Kinetin and TDZ on frequency of multiple shooting from plumule junction explants indicating height and nature of regenerants for cultivar IPA-3088. Date recorded from 10 replicates of explants for each concentration after 4 weeks of culture [S= Shoot (Size more than 1 cm), SB= Shoot buds (size up to 1cm) and SP= shoot primordia].

Hormone concentration (mgL^{-1})	Multiple shooting (%)	Length of regenerants in cm (Mean ± S.D.)	Nature of regenerants and their occurrence (%)
BAP			
0.5	100	1.0±0.3	12.24%SP+57.14%SB+30.61%S
1.0	50	1.8±1.5	29.62%SP+ 100%S
1.5	40	4.8±2.0	100%S
2.0	100	2.1±1.0	22.72%SB+ 77.27%S
2.5	90	3.8±1.7	9.25%SB+ 90.74%S
3.0	100	5.6±1.2	100%S
3.5	90	2.3±0.7	14.63%SB+ 85.36%S
4.0	60	2.9±0.9	24.39%SB+ 75.61%S
Kinetin			
0.5	90	11.4±2.3	100%S
1.0	90	6.7±0.9	100%S
1.5	100	4.3±0.9	100%S
2.0	100	6.2±0.8	100%S
2.5	100	4.6±0.9	100%S
3.0	50	4.7±1.4	100%S

Table 4. Contd

3.5	80	7.4±0.9	100%S
4.0	60	4.7±1.5	100%S
TDZ			
0.05	100	3.3±0.2	100%S
0.10	100	1.6±0.4	0.98%SP+ 99%S
0.15	100	1.3±0.6	3.23%SP+ 25.81%SB+ 70.97%S
0.20	100	2.7±1.1	2.53%SP+ 97.47%S
0.25	100	1.0±0.3	4.08%SP+ 63.27%SB+ 32.65%S
0.30	100	1.5±0.6	1.94%SP+ 98.06%S
0.35	70	0.7±0.3	23.53%SP+ 70.59%SB+ 5.8%S
0.40	100	1.3±0.8	18.18%SP+ 81.82%S

Table 5. Comparative response of plumule junction explant of different cultivars showing multiple shooting (Mean ± SD in %). Average was taken from multiple shooting responses under different concentration of each growth regulator for each cultivar.

Name of cultivars	**% Multiple shooting (Mean±SD)**		
	BAP	**Kinetin**	**TDZ**
IPA-2013	74±30	63±48	38±48
IPA-3088	79±23	84±18	90±18
Pusa-9	36±38	44±47	83±24
IPA-34	88±33	4.0±9.0	38±48
IPA-204	44±39	20±36	90±26
IPA-242	59±32	6.0±17	19±35
T-7	63±38	0	83±19
IPA-61	83±24	0	38±48
IPA-337	60±42	8.0±20	41±44
IPA-341	63±48	0	13±33
IPA-98-3	19±35	0	100±0.0

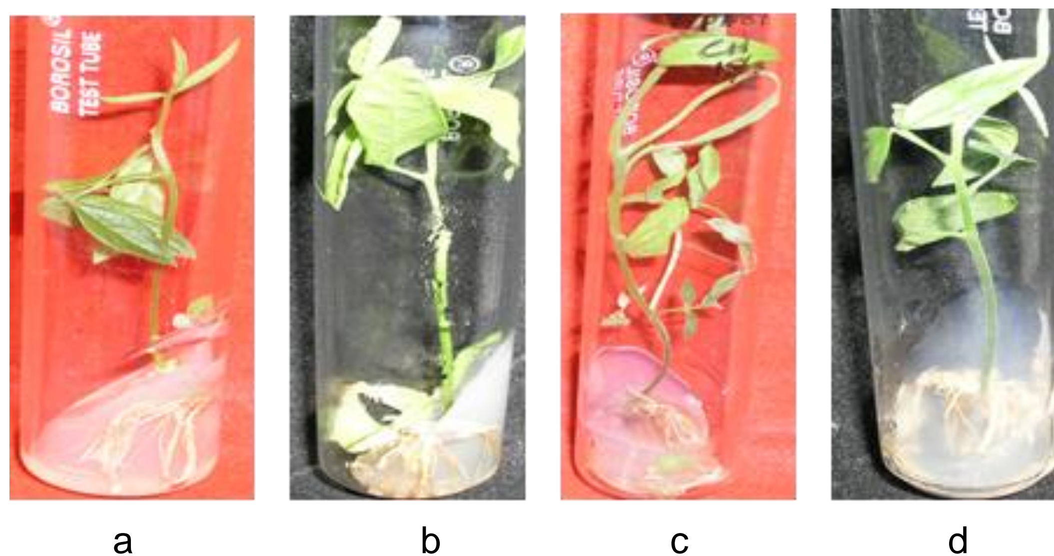

Figure 4. Rooting response for multiple shoot buds from plumule junction explants of different cultivars of pigeon pea on MS media supplemented with different concentration of NAA. **(a)** IPA-3088 (0.2mgL^{-1}); (**b)** Pusa-9 (0.2 mgL^{-1}); (c) IPA-34 (0.1 mgL^{-1}); **(d)** IPA-242 (0.2 mgL^{-1}).

Table 6. Rooting responses of *in vitro* regenerated shoots from plumule junction explants under different concentrations of NAA. Date recorded after 4 weeks of culture with 10 replicates for each treatment and experiment was repeated twice.

Cultivars	NAA (0.1 mgL^{-1})		NAA (0.2 mgL^{-1})		NAA (0.3 mgL^{-1})	
	% of rooting	Number of primary roots Mean±S.D.	% of rooting	Number of primary roots Mean±S.D	% of rooting	Number of primary roots Mean±S.D
IPA-2013	50	0.5±0.5	100	5.0±0.0	NR	NR
IPA-3088	70	0.7±0.5	100	11.6±0.9	80	3.2±1.6
Pusa-9	90	3.6±1.2	100	11.3±0.9	NR	NR
IPA-34	100	10.5±0.5	100	6.6±2.0	NR	NR
IPA-204	NR	NR	100	3.6±0.5	NR	NR
IPA-242	100	1.0±0.0	100	12.2±0.9	50	0.5±0.5
T-7	NR	NR	100	1.0±0.0	NR	NR
IPA-61	100	5.0±0.4	NR	NR	NR	NR
IPA-337	NR	NR	NR	NR	NR	NR
IPA-341	100	5.1±0.5	100	2.7±0.5	NR	NR
IPA-98-3	NR	NR	100	4.0±0.6	NR	NR

Figure 5. Acclimatized plants of cultivar IPA-3088.

three different concentrations (0.1, 0.2 and 0.3 mgL^{-1}) of IAA and IBA. The rooting response was better at 0.1 mgL^{-1} concentration of IAA with overall 70 to 100% rooting in few of the cultivars. The cultivar IPA-204 showed best response for rooting with 0.1 mgL^{-1} IAA. The rooting response was very poor in the presence of IBA and only two cultivars IPA-204 and IPA-61 showed rooting response when subjected to 0.1 mg/l IBA with 90 and 50% of rooting respectively. The percentage acclimatization of multiple shoot buds derived from plumule junction explants with proper rooting in soil ranged from 40 to 85% with cultivar IPA-3088 showing maximum percentage of acclimatization (Figure 5).

In most of the earlier reports two different media with different hormones were used for shoot bud induction and elongation. Shoot bud induction was attempted on MS media supplemented with different concentration of BAP (Geetha et al., 1998; Shiva Prakesh et al., 1994) or TDZ (Eapen et al., 1998) while for elongation, different media containing IAA (Shiva Prakesh et al., 1994) or Gibberelic acid (Mohan and Krishnamurthy, 1998) or in combination of both (Eapen et al., 1998) or lower concentration of BAP and NAA (Geetha et al., 1998) has been used. In the present study same media was used for induction and elongation similar to what has been reported by Singh et al. (2002). In this study, the direct organogenesis protocol involving induction and elongation of shoot buds in same media supplemented with BAP, Kinetin and TDZ might be preferred as the plantlets developed directly without an intervening callus phase, which minimizes the chance of somaclonal variations in the regenerants. Further, our work has shown the comparative account of multiple shoot regeneration from eleven cultivars using one type of explant in order to identify the most responsive cultivar for further transformation use. The results clearly indicates superiority of IPA-3088 which is a candidate for choice for further investigation instead of trying with poorly responding cultivars which may end up with recalcitrance for plant regeneration.

Conclusion

The *in vitro* multiple shoot bud induction and regeneration among 11 Indian cultivars of pigeon pea using plumule junction explants under the influence of three different

hormones has been studied. The genotype dependent variation was observed. The cultivar IPA-3088 is found to be highly efficient for multiple shoot but induction and *in vitro* regeneration among these cultivars at lower concentration of TDZ results in a maximum of 20 shoots buds per explants. This cultivar should be a suitable candidate for developing genetic transformation protocols with desired agronomic traits using either *Agrobacterium* or microprojectile based methods of transformation.

Conflict of Interests

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

ACKNOWLEDGMENTS

The authors wish to acknowledge Dr. B.B Singh and S.D. Dubey, Indian Institute of Pulses Research, Kanpur, India, Dr. Harpal Singh, Punjab Agricultural University, Gurdaspur, Director Experiment Station, G.B Pant University of Agriculture and Technology, Pantnagar for providing the seeds of pigeon pea cultivars used in the present study. The authors are also thankful to Director, NEERI, CSIR, Nagpur for providing Plant Tissue Culture Lab facility to some conduct part of the work in NEERI, Nagpur.

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Vol. 13(41), pp. 4070-4080, 8 October, 2014
DOI: 10.5897/AJB2014.13726
Article Number: BE3AEF447975
ISSN 1684-5315
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African Journal of Biotechnology

Full Length Research Paper

Control of root-knot nematode by using composted sawdust in tomato root

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Received 14 February, 2014; Accepted 15 August, 2014

The effect of composted sawdust at different concentrations (0, 10, 20... 100% v/v in soil) on tomato plant growth and pigments were investigated with or without the presence of root-knot nematode, *Meloidogyne javanica* at different inoculum levels (0, 1000, 2000, 3000, 4000, 5000) in clay pots (having diameter 30 cm and depth 60 cm). An increase in the composted sawdust concentration in the field soil progressively increased the availability of chlorides, sulphates, carbonates, bicarbonates, P, K, Mg, Mn, Cu, Zn and Fe. However, reverse trend was evaluated in nitrogen content of the soil with gradual increment in sawdust. Different physical properties such as porosity, pH, conductivity, water holding and cation exchange capacity also increased gradually with gradual sawdust amendment in the soil. Sawdust application enhanced the plant growth as well as leaf pigments in both nematode infected as well as non-infected tomato plants, being maximum in the soil containing 30% composted sawdust. Growth and leaf pigments also showed reductions with respect to increase in nematode inoculum density compared to nematode unionculated plants (that is, controls). However, least amount of tomato growth and leaf pigments were found at 3000 nematode inoculum level. Sawdust treatments favorably affect the root invasion by root-knot nematode juveniles (J2 and J3 + J4) and galls up to 30% but adversely affected onward treatments although, a gradual increase in sawdust concentration in the soil would correspondingly decrease the number of egg masses and eggs per egg mass (that is, fecundity) of the root-knot nematodes. All the above said nematode parameters were also improved with all considered nematode inoculum levels but 3000 was the optimum level for them. After visualizing the results, it can be suggested that 30% composted sawdust was the most economical level as it enhances the growth and pigments irrespective of the presence or absence of root-knot nematode. At the same time, it also controls the root-knot nematodes in particularly in 30% onward dust amendments.

Key words: Meloidogyne javanica, nematode,sawdust.

INTRODUCTION

Sawdust, composed of fine particles of wood are the by-product of sawmills. It litters into the surrounding area and accumulates as fine particles in the soil. It adds some harmful and toxic leachates to the soil and thus to the water

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system. The water born bacteria digest organic material present in leachates and use up much of the available oxygen and increase the biochemical oxygen demand of the water. However, some reports are also available regarding the improvement in nutritional pool of the soil through composted sawdust additions (Obasi et al., 2013). It also improves some of the physico-chemical properties of the soil such as soil porosity (Imre et al., 2011). Recently, Hassan et al. (2010) have reported the growth promoting effects of sawdust on tomato plants.

Root-knot nematode, *Meloidogyne* species happens to be an important pest parasite on different vegetable plants in tropical as well as subtropical countries. Root-knot nematode attacks several kind of crop all over the world due their wide host range. The nematode presence may cause the damage from 25 to 60% in yield (Akhtar et al., 2012). The nematode density is also a matter of concern to the crop growth and yield (Hong et al., 2011). Although, some reports are available regarding the inhibitory effect of sawdust on nematodes due to formation of phenolic compounds through the decomposition of sawdust (Kokalis-Burelle et al.,1994). However, it is not possible to draw systematically available information based on the interaction of nematodes with variable density in the presence of different sawdust concentrations. So far there are no available literatures with respect to meticulous utilization of sawdust to control the root-knot nematode in their parasitic or nonparasitic phases. So here an experiment was designed to assess the potentiality of sawdust as the nematicide and/or fertilizer, which can be utilized for the management of the root-knot nematode on one hand as well as crop growth improvement on the other.

MATERIALS AND METHODS

Sawdust used in this experiment was collected from sawmill situated at Quarsi, the suburb of Aligarh (U.P.). Sawdust was composted for three months in a dug out pit for decomposition. The composted sawdust and field loamy soil (procured from field) were sun dried for a week and then mixed in requisite quantities to obtain the different sawdust levels (that is, 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% v/v) and filled (3 kg/pot) in clay pots having 30 cm upper diameters. The pots were then sterilized in autoclave maintained at 120°C temperature for 12 to 19 min at 20 lb pressure.

Physio-chemical analysis

The physio-chemical characters of soil samples, with or without sawdust, were determined prior to seedling transplantation. But before analyzing such properties, fine particles of each sample were collected by passing them in a fine sieve. Porosity and water holding capacity were determined through hydrometry, pH by pH meters and CEC by analytical method. Carbonates and bicarbonates were determined by using the proper method. Nitrogen and phosphorus contents were also estimated by utilizing the appropriate technique. Zinc, copper, iron and manganese were determined by Diethylene triamine penta acetic acid (DTPA) method, potassium by ammonium acetate method and magnesium by mixed acid digestion method.

Plant and nematode culture

Two-week-old seedlings of tomato, already grown in autoclaved field soil, were transplanted to the pots having sawdust and soil mixture in different proportions. One week after planting the seedlings, the pots which were designated to receive *M. javanica* were inoculated with freshly hatched second stage juveniles (J_2) of the root-knot nematode. Pure culture of J_2 was obtained from a single egg mass culture, which was done earlier in order to obtain the sufficient inoculum by culturing and sub culturing on the egg plants. For inoculation, the nematode suspension of 1 ml water containing 1000 juvenile (counting was done in counting dish) was added in holes made in the soil around a seedling as per treatment. Each treatment was replicated five times and pots were arranged in Complete Randomized Block Design (CRBD) on greenhouse benches (30±2°C) of Botanical Garden of D.S. College, Aligarh. After 90 days of growth, tomato plants were harvested for the measurement of various parameters.

Estimation of plant growth

A few hours before termination of the experiment, an excess amount of water was added to the pots in order to soften the soil so that tomato plants could be uprooted softly without doing excessive loss to the roots. Uprooted plants were brought to the laboratory in the polypacks and lengths, fresh and dry weight of shoot and root were determined through standard procedure. The dry weight was however, determined after drying the shoot and root in hot air oven at 80°C for a full day and night.

Estimation of pigments

The interveinal tissue from fresh leaves (1 g per plant) of unharvested plants was ground in 80% actone and filter through two Whatman No. 1 filter papers. The filtrate was use to determine carotenoid (MacLachlan and Zalik, 1963) and chlorophyll contents (MacKinney, 1941).

Number of galls, egg masses, juveniles (J_2 and $J_3 + J_4$) and fecundity

At termination of the experiment, roots of harvested plants were washed under tap water for examination of nematode penetration level. Root samples (1 g) from different nematode treated plants were stained with acid fuchsin and lactophenol and pressed between two glass slides and examined under the compound microscope for second, third and fourth stage (J_2 and $J_3 + J_4$) juveniles of root-knot nematode in the roots.

Root galling and reproduction of the nematodes were determined by determining the number of galls and egg masses in tomato roots of nematode treated plants. The harvested plants were washed under tap water and examined for the presence of galls. Numbers of galls were counted in plant roots through naked eyes. Roots were immersed in an aqueous solution of phloxin B (0.15 g/lit tap water) for 15 min to stain the egg masses and then egg masses were counted. For the estimation of fecundity 10 egg masses shaked vigorously in 5.25 NaOCl solutions. The eggs were separated from egg masses and collected over 5000 mesh sieve. From the sieve the eggs were transferred to a beaker and 0.35% acid fuchsin (in 25% lactic acid) was added to 20 to 25 ml of suspension with boiling for 1 min for staining the eggs. After cooling, the eggs were counted and tabulated as eggs per egg mass.

Statistical analysis

One factorial analysis was employed for the evaluation of different

Table 1. Effect of sawdust on physio-chemical properties of soil.

Sawdust concentration (%)	Characteristic																
	Porosity	WHC	pH	Conductivity	CEC	Sulphate (%)	Carbonate (%)	Bicarbonate (%)	Chloride (%)	Nitrogen (%)	Phosphorus (%)	Potassium (%)	Magnesium (%)	Manganese (µg/g)	Copper (µg/g)	Zinc (µg/g)	Iron (µg/g)
0	48.5	33.7	6.2	3.8	5.7	4.62	0.45	0.69	0.31	0.16	0.05	0.24	0.07	18.1	2.12	1.15	7.48
10	51.7ns	42.1*	6.8*	3.9ns	5.9ns	4.98*	0.67*	1.27*	0.86*	0.15*	0.09*	0.26ns	0.10*	163.5*	8.35*	10.50*	11.640*
20	54.7*	46.4*	5.7*	4.0ns	6.0ns	5.14*	0.98*	1.79*	1.08*	0.14*	0.13*	0.29*	0.15*	274.3*	9.34*	23.70*	132.40*
30	63.7*	48.8*	7.0*	4.1*	6.0ns	5.56*	1.16*	2.14*	1.47*	0.12*	0.24*	0.33*	0.27*	368.4*	16.70*	28.50*	156.70*
40	65.2*	56.4*	7.3*	4.2*	6.1*	5.76*	1.19*	2.19*	1.51*	0.10*	0.28*	0.39*	0.29*	371.1*	17.30*	32.20*	165.30*
50	66.7*	66.9*	7.4*	4.6*	6.1*	5.89*	1.20*	2.38*	1.56*	0.06*	0.29*	0.45*	0.32*	382.4*	17.90*	33.80*	169.70*
60	67.3*	78.6*	7.6*	4.8*	6.2*	6.37*	1.21*	2.41*	1.58*	0.04*	0.31*	0.49*	0.34*	384.6*	18.10*	34.70*	172.10*
70	68.4*	81.2*	7.7*	4.8*	6.2*	6.81*	1.21*	2.43*	1.62*	0.00*	0.32*	0.56*	0.38*	389.1*	18.20*	35.10*	177.40*
80	71.3*	83.4*	7.7*	4.9*	6.3*	6.88*	1.32*	2.51*	1.66*	0.00*	0.38*	0.69*	0.42*	392.2*	18.60*	36.80*	179.20*
90	73.4*	86.4*	7.8*	5.1*	6.3*	6.90*	1.34*	2.56*	1.67*	0.00*	0.41*	0.80*	0.46*	421.2*	18.90*	37.40*	185.20*
100	82.1*	87.9*	7.8*	5.1*	6.5*	6.92*	1.37*	2.68*	1.75*	0.00*	0.47*	0.98*	0.51*	441.3*	19.50*	38.50*	186.70*
LSD at 5%	3.42	3.64	0.383	0.236	0.322	0.316	0.060	0.116	0.078	0.007	0.017	0.031	0.019	19.24	0.877	1.69	8.75

* = data significant with 0 inoculation level and 0% dust concentration at P=0.05; ns = not significant; # = data significant within a column at P=0.05; @ = data significant in a row at P = 0.05.

physio-chemical property data of soil with or without sawdust. The least significant difference (LSD) was calculated at P=0.05 by subjecting the data to Anova Table.

The data of tomato growth, leaf pigments and different root-knot disease parameters were analyzed by two factor analysis. The data are of two factors in which sawdust was considered as factor one (F_1) while different nematode inoculation levels were considered as factor two (F_2). The LSD was thus calculated separately for these factors along with their interactive LSD at P=0.05.

RESULTS

Physico-chemical properties

Data shows (Table 1) that the pH, conductivity, cation exchange capacity (CEC), water holding capa-city (WHC) and porosity were increased gradually with gradual increase in the sawdust level. Sulphate, carbonate, bicarbonate and chloride contents of sawdust were 49, 204, 288 and 464% compared to the field soil; thereby all were increased linearly with increase in sawdust concentration of field soil. Nitrogen contents were 0.160% in the field soil but were undetectable in sawdust particularly onward to 60% levels. A gradual increase in different metals (magnesium, manganese, copper, zinc and iron) was also observed in ever increasing level of sawdust.

Plant growth and photosynthetic pigments

Plant growth in terms of length, fresh and dry weight of shoot and root and leaf photosynthetic pigments in terms of chlorophyll a, chl b, total chl and carotenoid contents were enhanced upto 30% sawdust level as evident from the data presented in Tables 2 to 6. Above 30% level, sawdust was however, proved detrimental to plant growth and photosynthetic pigments. The least value of these parameters was found in pure dust grown tomato plants. All growth related parameters including pigments were suppressed gradually with increase in the nematode density level from none (that is, control) to top levels (that is, 5000 inoculum level). However the maximum suppression to them was occurred at 3000 nematode level but this suppression was slightly masked at 4000 and 5000 nematode inoculation levels compared to 3000 level, although it was still

Table 2. Effect of sawdust on length of shoot and root (cm) of tomato plants.

Sawdust concentration (%)	Parameters	Inoculation levels						
		0	1000	2000	3000	4000	5000	Mean
0	Shoot	38.24	36.32^{ns}	32.80*	26.14*	27.08*	27.02*	31.27
	Root	18.58	17.36^{ns}	16.78^{ns}	14.72^{ns}	15.80^{ns}	15.00^{ns}	16.37
10	Shoot	38.80^{ns}	36.70^{ns}	33.10*	26.40*	28.20*	27.40*	31.77^{ns}
	Root	18.50^{ns}	17.70^{ns}	17.20^{ns}	15.20^{ns}	16.20^{ns}	15.60^{ns}	16.73^{ns}
20	Shoot	39.20^{ns}	37.10^{ns}	33.40*	26.70*	28.60*	27.80*	32.13$^{\#}$
	Root	19.40^{ns}	18.20^{ns}	17.70^{ns}	15.60^{ns}	16.60^{ns}	16.20^{ns}	17.28$^{\#}$
30	Shoot	39.60^{ns}	37.60^{ns}	33.90*	27.00*	29.00*	28.40*	32.58$^{\#}$
	Root	20.00^{ns}	18.60^{ns}	18.30^{ns}	16.20^{ns}	17.00^{ns}	16.80^{ns}	17.82$^{\#}$
40	Shoot	36.10^{ns}	34.60*	30.30*	23.50*	24.30*	23.70*	28.75$^{\#}$
	Root	16.50^{ns}	15.30^{ns}	14.82^{ns}	12.70^{ns}	13.60^{ns}	12.90^{ns}	14.30$^{\#}$
50	Shoot	29.80*	26.80*	22.50*	19.00*	22.70*	21.20*	23.67$^{\#}$
	Root	15.30^{ns}	14.10^{ns}	12.90^{ns}	10.80^{ns}	11.86^{ns}	11.20^{ns}	12.69$^{\#}$
60	Shoot	18.70*	17.60*	16.10*	14.00*	15.30*	15.00*	16.12$^{\#}$
	Root	12.70^{ns}	11.50^{ns}	10.00^{ns}	8.60^{ns}	9.50^{ns}	9.00^{ns}	10.22$^{\#}$
70	Shoot	13.90*	11.50*	10.80*	9.20*	10.50*	9.80*	10.95$^{\#}$
	Root	10.40^{ns}	10.00^{ns}	9.10^{ns}	8.50^{ns}	9.00^{ns}	8.80^{ns}	9.30$^{\#}$
80	Shoot	10.60*	8.90*	8.40*	7.50*	8.00*	7.50*	8.48$^{\#}$
	Root	8.70^{ns}	7.20^{ns}	6.80^{ns}	5.90^{ns}	6.30^{ns}	6.20^{ns}	6.85$^{\#}$
90	Shoot	8.80*	8.00*	7.70*	6.80*	7.50*	7.10*	7.65$^{\#}$
	Root	6.50^{ns}	6.00^{ns}	5.60^{ns}	5.00^{ns}	5.50^{ns}	5.20^{ns}	5.63$^{\#}$
100	Shoot	7.70*	7.20*	6.80*	6.40*	6.60*	6.60*	6.88$^{\#}$
	Root	5.00^{ns}	4.10^{ns}	3.90^{ns}	3.10^{ns}	3.60^{ns}	3.50^{ns}	3.87$^{\#}$
Mean	Shoot	25.59	23.85$^{@}$	21.44$^{@}$	17.51$^{@}$	18.89$^{@}$	18.32$^{@}$	
	Root	13.78	12.73$^{@}$	12.10$^{@}$	10.57$^{@}$	11.36$^{@}$	10.95$^{@}$	
LSD at 5%	Shoot	Dust	0.700	Nematode inoculation	0.947	Interaction	2.321	
	Root	Dust	0482	Nematode inoculation	0.653	Interaction	NS	

* = data significant with 0 inoculation level and 0% dust concentration at *P*=0.05; ns = Not significant; # = data significant within a column at *P* = 0.05; @ = data significant in a row at *P* = 0.05.

greater than controls.

Root-knot nematode caused significant suppressions to plant growth and leaf pigments in sawdust treated and untreated plants. However, the suppressive effects of the nematode gradually decreased with gradual increase in the sawdust concentration of the soil.

Root-knot disease

The juvenile (J_2 and J_3+J_4) invasion was significantly impaired by different sawdust concentrations. This is evident from Table 7 and 10; there were increase upto 30% levels but decreased onward with dust additions. Likewise changes were recorded in root galling with respect to sawdust. As it was significantly improved upto 30% sawdust amendment (Table 8). There number suppressed in 30% onward sawdust treatments with the minimum number in 60% amendments. Egg masses and fecundity of the nematodes were suppressed gradually with gradual increase in sawdust concentration. All

Table 3. Effect of sawdust on fresh weight of shoot and root (g) of tomato plants.

Sawdust concentration	Parameters	Inoculation levels						
		0	1000	2000	3000	4000	5000	Mean
0	Shoot	35.20	33.08^{ns}	31.50^{ns}	27.44^{ns}	29.22^{ns}	28.64^{ns}	30.85
	Root	13.50	12.90^{ns}	11.80*	10.10*	11.70*	11.20*	11.87
10	Shoot	36.70^{ns}	34.20^{ns}	32.10^{ns}	28.40^{ns}	30.70^{ns}	29.30^{ns}	31.90$^{\#}$
	Root	13.90^{ns}	13.40^{ns}	11.90*	10.40*	12.00*	11.80*	12.17$^{\#}$
20	Shoot	37.90^{ns}	35.40^{ns}	33.40^{ns}	29.70^{ns}	31.50^{ns}	30.20^{ns}	33.02$^{\#}$
	Root	14.50*	13.80^{ns}	12.90*	10.72*	12.40*	12.20*	12.59$^{\#}$
30	Shoot	38.50^{ns}	36.70^{ns}	33.90^{ns}	30.10^{ns}	32.70^{ns}	31.00^{ns}	33.82$^{\#}$
	Root	14.90*	14.26^{ns}	13.40^{ns}	11.50	12.80^{ns}	12.50*	13.23$^{\#}$
40	Shoot	35.48^{ns}	33.20^{ns}	30.10^{ns}	27.50^{ns}	29.40^{ns}	28.80^{ns}	30.75$^{\#}$
	Root	14.20^{ns}	13.30^{ns}	12.80^{ns}	11.30*	12.40*	11.90*	12.65$^{\#}$
50	Shoot	30.20^{ns}	28.70^{ns}	26.70^{ns}	24.80^{ns}	26.70^{ns}	25.70^{ns}	27.13$^{\#}$
	Root	12.66^{ns}	11.20*	10.80*	9.90*	10.40*	10.20*	10.86$^{\#}$
60	Shoot	27.66^{ns}	25.30^{ns}	23.20^{ns}	21.60^{ns}	22.90^{ns}	22.40^{ns}	23.84$^{\#}$
	Root	11.10*	9.10*	8.10*	6.72*	7.70*	7.10*	8.39$^{\#}$
70	Shoot	20.50^{ns}	18.80^{ns}	16.50^{ns}	14.30^{ns}	15.70^{ns}	15.00^{ns}	16.80$^{\#}$
	Root	8.60*	7.00*	6.80*	6.10*	6.60*	6.40*	6.92$^{\#}$
80	Shoot	18.90^{ns}	16.50^{ns}	14.10^{ns}	11.20^{ns}	13.50^{ns}	12.20^{ns}	14.40$^{\#}$
	Root	6.10*	5.80*	5.50*	5.00*	5.20*	4.80*	5.40$^{\#}$
90	Shoot	14.70^{ns}	12.00^{ns}	10.30^{ns}	7.30^{ns}	9.00^{ns}	8.70^{ns}	10.33$^{\#}$
	Root	5.80*	5.20*	4.70*	4.20*	4.50*	4.50*	4.82$^{\#}$
100	Shoot	8.80^{ns}	6.90^{ns}	6.50^{ns}	5.50^{ns}	6.00^{ns}	5.80^{ns}	6.58$^{\#}$
	Root	4.50*	4.00*	3.60*	3.20*	3.40*	3.40*	3.68$^{\#}$
Mean	Shoot	27.69	25.53$^{@}$	23.48$^{@}$	20.71$^{@}$	22.48$^{@}$	21.61$^{@}$	
	Root	10.89	10.04$^{@}$	9.17$^{@}$	8.10$^{@}$	9.01$^{@}$	8.73$^{@}$	
LSD at 5%	Shoot	Dust	0.916	Nematode inoculation	1.241	Interaction	NS	
	Root	Dust	0.260	Nematode inoculation	0.353	Interaction	0.864	

* = data significant with 0 inoculation level and 0% dust concentration at *P*=0.05; ns = Not significant; # = data significant within a column at *P* = 0.05; @ = data significant in a row at *P* = 0.05.

these nematode parameters were found nil in 70, 80, 90 and 100% sawdust additions (Tables 9 and 10).

DISCUSSION

Sawdust has gradually improved the growth and photosynthetic pigments of the tomato plants up to 30% amendments. Some reports are also available with regards to positive effects of sawdust on plant growth (Hassan et al., 2010). Improvement in different physio-chemical properties of the soil with sawdust additions (Table 1) are in concurrence with the earlier work (Obasi et al., 2013). The optimization in the soil properties has occurred most appropriately at 30% dust addition as evident from the maximization of tomato's growth and pigments in such treatments. Reverse effects of sawdust were observed on the growth and pigments of tomato plants beyond 30% amendments. At higher levels, accumulation of heavy metals beyond threshold

Table 4. Effect of sawdust on dry weight of shoot and root (g) of tomato plants.

Sawdust concentration	Parameters	Inoculation levels						
		0	1000	2000	3000	4000	5000	Mean
0	Shoot	7.70	7.41^{ns}	6.60^{ns}	5.94^{ns}	6.45^{ns}	6.27^{ns}	6.73
	Root	2.90	2.80^{ns}	2.70^{ns}	2.30^{ns}	2.52^{ns}	2.43^{ns}	2.61
10	Shoot	8.00^{ns}	7.80^{ns}	7.30^{ns}	6.80^{ns}	7.20^{ns}	7.00^{ns}	$7.35^{\#}$
	Root	3.10^{ns}	3.00^{ns}	2.90^{ns}	2.50^{ns}	2.80^{ns}	2.60^{ns}	$2.82^{\#}$
20	Shoot	8.70^{ns}	8.20^{ns}	7.90^{ns}	7.30^{ns}	7.80^{ns}	7.70^{ns}	$7.93^{\#}$
	Root	3.30^{ns}	3.20^{ns}	3.10^{ns}	2.94^{ns}	3.00^{ns}	3.00^{ns}	$3.09^{\#}$
30	Shoot	9.30^{ns}	8.90^{ns}	8.50^{ns}	8.10^{ns}	8.40^{ns}	8.20^{ns}	$8.57^{\#}$
	Root	3.50^{ns}	3.40^{ns}	3.20^{ns}	3.10^{ns}	3.20^{ns}	3.10^{ns}	$3.25^{\#}$
40	Shoot	7.90^{ns}	7.20^{ns}	6.90^{ns}	6.10^{ns}	6.80^{ns}	6.60^{ns}	6.92^{ns}
	Root	3.10^{ns}	3.00^{ns}	2.70^{ns}	2.30^{ns}	2.60^{ns}	2.40^{ns}	2.68^{ns}
50	Shoot	7.20^{ns}	6.90^{ns}	6.40^{ns}	5.70^{ns}	6.20^{ns}	6.10^{ns}	$6.42^{\#}$
	Root	3.00^{ns}	2.60^{ns}	2.50^{ns}	2.20^{ns}	2.40^{ns}	2.30^{ns}	2.50^{ns}
60	Shoot	6.80^{ns}	6.40^{ns}	6.00^{ns}	5.40^{ns}	5.80^{ns}	5.60^{ns}	$6.00^{\#}$
	Root	2.80^{ns}	2.20^{ns}	2.00^{ns}	1.50^{ns}	1.80^{ns}	1.60^{ns}	$1.98^{\#}$
70	Shoot	6.30^{ns}	6.00^{ns}	5.50^{ns}	5.10^{ns}	5.40^{ns}	5.20^{ns}	$5.58^{\#}$
	Root	2.40^{ns}	2.00^{ns}	1.70^{ns}	1.30^{ns}	1.50^{ns}	1.50^{ns}	$1.73^{\#}$
80	Shoot	5.00^{ns}	4.80^{ns}	4.60^{ns}	4.20^{ns}	4.40^{ns}	4.40^{ns}	$4.57^{\#}$
	Root	2.00^{ns}	1.80^{ns}	1.60^{ns}	1.10^{ns}	1.40^{ns}	1.20^{ns}	$1.52^{\#}$
90	Shoot	3.70^{ns}	3.40^{ns}	3.20^{ns}	2.50^{ns}	3.00^{ns}	2.80^{ns}	$3.10^{\#}$
	Root	1.50^{ns}	1.30^{ns}	1.10^{ns}	0.90^{ns}	1.00^{ns}	1.00^{ns}	$1.13^{\#}$
100	Shoot	2.20^{ns}	2.10^{ns}	2.04^{ns}	1.70^{ns}	1.90^{ns}	1.90^{ns}	$1.97^{\#}$
	Root	1.20^{ns}	1.00^{ns}	1.00^{ns}	0.80^{ns}	1.00^{ns}	1.00^{ns}	$1.00^{\#}$
Mean	Shoot	6.62	$6.28^{@}$	$5.90^{@}$	$5.35^{@}$	$5.76^{@}$	$5.62^{@}$	
	Root	2.62	$2.39^{@}$	$2.23^{@}$	$1.90^{@}$	$2.11^{@}$	$2.01^{@}$	
LSD at 5%	Shoot	Dust	0.189	Nematode inoculation	0.256	Interaction	NS	
	Root	Dust	0.138	Nematode inoculation	0.187	Interaction	NS	

* = data significant with 0 inoculation level and 0% dust concentration at P=0.05; ns = Not significant; # = data significant within a column at P = 0.05; @ = data significant in a row at P = 0.05.

limit for plants can be advocated as reason behind such adversaries on growth fronts. Since nitrogen being an integral part of the chlorophyll (Javedi, 2014) so nitrogen immobilization due to its more deficiency at higher dust amendments could also be interpreted as healthy reason behind such poor growth and pigmentation of tomato. Higher doses of sawdust are reported to be phytotoxic to tomato growth and yield (Siddiqui and Alam, 1990).

The reduction in growth and leaf pigments of tomato plants of about 3000 root-knot nematode inoculum levels could be due to the formation of galls through hyperplastic and hypertropic phenol-menon. The sedentary females of *M. javanica* obtained food from such galls. The nutrients

Table 5. Effect of sawdust on chlorophyll ‘a’ and chlorophyll ‘b’ (μg/g) of tomato leaves.

Sawdust concentration	Parameters	Inoculation levels						
		0	1000	2000	3000	4000	5000	Mean
0	Chl.a	520.0	507.0^{ns}	498.0^{ns}	480.0^{ns}	493.0^{ns}	490.0^{ns}	498.00
	Chl.b	235.00	227.00*	219.00*	203.00*	215.00*	212.00*	218.50
10	Chl.a	535.0^{ns}	525.0^{ns}	517.0^{ns}	502.0^{ns}	512.0^{ns}	508.0^{ns}	516.5$^{\#}$
	Chl.b	238.00*	231.00*	226.00*	212.00*	222.00*	218.00*	224.50$^{\#}$
20	Chl.a	547.0^{ns}	535.0^{ns}	527.0^{ns}	515.0^{ns}	524.0^{ns}	520.0^{ns}	528.0$^{\#}$
	Chl.b	241.00*	237.00^{ns}	233.00^{ns}	220.00*	229.00*	225.00*	230.83$^{\#}$
30	Chl.a	560.0^{ns}	549.0^{ns}	538.0^{ns}	529.0^{ns}	535.0^{ns}	530.0^{ns}	540.2$^{\#}$
	Chl.b	245.00*	241.00*	238.00*	231.00*	235.00^{ns}	233.00^{ns}	237.17$^{\#}$
40	Chl.a	512.0^{ns}	500.0^{ns}	472.0^{ns}	450.0^{ns}	465.0^{ns}	457.0^{ns}	476.0$^{\#}$
	Chl.b	236.00^{ns}	230.00*	223.00*	215.00*	221.00*	218.80*	223.97$^{\#}$
50	Chl.a	480.0^{ns}	468.0^{ns}	457.0^{ns}	447.0^{ns}	454.0^{ns}	451.0^{ns}	459.5$^{\#}$
	Chl.b	225.00*	219.00*	211.00*	201.00*	208.00*	205.00*	211.50$^{\#}$
60	Chl.a	450.0^{ns}	436.0^{ns}	425.0^{ns}	417.0^{ns}	420.0^{ns}	418.2^{ns}	427.7$^{\#}$
	Chl.b	213.00*	205.00*	193.00*	182.00*	190.20*	185.00*	194.70$^{\#}$
70	Chl.a	410.0^{ns}	395.0^{ns}	377.0^{ns}	353.0^{ns}	370.0^{ns}	365.0^{ns}	378.3$^{\#}$
	Chl.b	202.00*	195.00*	182.00*	170.00*	180.00*	176.00*	184.17$^{\#}$
80	Chl.a	350.0^{ns}	325.0^{ns}	307.0^{ns}	282.0^{ns}	302.0^{ns}	290.0^{ns}	309.3$^{\#}$
	Chl.b	184.00*	172.00*	163.00*	152.00*	161.00*	156.00*	164.67$^{\#}$
90	Chl.a	300.0^{ns}	272.0^{ns}	252.0^{ns}	227.0^{ns}	246.0^{ns}	234.8^{ns}	255.3$^{\#}$
	Chl.b	146.00*	138.00*	127.00*	118.00*	124.00*	120.00*	128.83$^{\#}$
100	Chl.a	110.0^{ns}	98.0^{ns}	86.0^{ns}	60.0^{ns}	79.0^{ns}	72.0^{ns}	84.2$^{\#}$
	Chl.b	112.00*	105.20*	97.00*	86.00*	93.00*	89.00*	97.03$^{\#}$
Mean	Chl.a	434.00	419.1$^{@}$	405.1$^{@}$	387.5$^{@}$	400.0$^{@}$	394.2$^{@}$	
	Chl.b	207.00	200.02$^{@}$	192.00$^{@}$	180.91$^{@}$	188.93$^{@}$	185.25$^{@}$	
LSD at 5%	Chl.a	Dust	9.698	Nematode inoculation	13.132	Interaction	NS	
	Chl.b	Dust	0.875	Nematode inoculation	1.185	Interaction	2.903	

* = data significant with 0 inoculation level & 0% dust concentration at P=0.05; ns = Not significant; # = data significant within a column at P = 0.05; @ = data significant in a row at P = 0.05.

continuously sucked by the nematode females, would subsequently be not available to plant to perform better at growth and leaf pigmentation front. Root-knot nematodes are also known to bring about an extensive alteration in the vascular tissues of the host plants therefore supply of water and nutrients are disturbed (Singh and Khan, 1999). The reduction occurred in plant growth and photosynthetic pigments was slightly masked at 4000 and 5000 compared to 3000 nematode inoculum levels although insignificant. Antagonistic interaction and/or intraspecific competition amongst the nematode (for food and space) could be extended as the reason beyond such insignificant improvements.

Table 6. Effect of sawdust on total chlorophyll and carotenoid (µg/g) of tomato leaves.

Sawdust concentration (%)	Parameters	Inoculation levels						
		0	1000	2000	3000	4000	5000	Mean
0	Chl	760.00	737.00*	720.00*	685.00*	712.00*	708.00*	720.33
	Carot.	4.24	3.90^{ns}	3.70^{ns}	3.40^{ns}	3.60^{ns}	3.60^{ns}	3.74
10	Chl	782.00*	762.00^{ns}	752.00*	721.00*	742.00*	733.00*	748.67$^{\#}$
	Carot.	4.30^{ns}	4.10^{ns}	3.90^{ns}	3.50^{ns}	3.80^{ns}	3.70^{ns}	3.88$^{\#}$
20	Chl	795.00*	783.00*	765.00*	743.00*	759.00^{ns}	751.00*	766.00$^{\#}$
	Carot.	4.50^{ns}	4.30^{ns}	4.10^{ns}	3.80^{ns}	4.00^{ns}	3.90^{ns}	4.10$^{\#}$
30	Chl	813.00*	794.00*	782.00*	765.00*	778.00*	769.00*	783.50$^{\#}$
	Carot.	4.90^{ns}	4.70^{ns}	4.40^{ns}	4.20^{ns}	4.30^{ns}	4.30^{ns}	4.47$^{\#}$
40	Chl	755.00*	737.00*	702.00*	673.00*	693.00*	683.00*	707.17$^{\#}$
	Carot.	4.80^{ns}	4.60^{ns}	4.30^{ns}	4.00^{ns}	4.20^{ns}	4.10^{ns}	4.33$^{\#}$
50	Chl	713.00*	691.00*	676.00*	653.00*	668.00*	663.00*	677.33$^{\#}$
	Carot.	4.40^{ns}	4.20^{ns}	4.02^{ns}	3.70^{ns}	3.90^{ns}	3.90^{ns}	4.02$^{\#}$
60	Chl	672.00*	700.00*	626.00*	607.00*	619.00*	609.00*	638.83$^{\#}$
	Carot.	4.10^{ns}	3.80^{ns}	3.66^{ns}	3.30^{ns}	3.50^{ns}	3.40^{ns}	3.63$^{\#}$
70	Chl	620.00*	597.00*	565.20*	532.00*	557.00*	549.00*	570.03*
	Carot.	3.90^{ns}	3.70^{ns}	3.40^{ns}	3.00^{ns}	3.20^{ns}	3.10^{ns}	3.38$^{\#}$
80	Chl	540.00*	507.00*	477.00*	441.00*	467.00*	453.00*	480.83$^{\#}$
	Carot.	3.50^{ns}	3.30^{ns}	3.20^{ns}	2.80^{ns}	3.00^{ns}	3.00^{ns}	3.13$^{\#}$
90	Chl	452.00*	418.00*	387.00*	352.40*	378.00*	364.00*	391.90$^{\#}$
	Carot.	3.00^{ns}	2.90^{ns}	2.60^{ns}	2.20^{ns}	2.40^{ns}	2.30^{ns}	2.57$^{\#}$
100	Chl	231.00*	209.00*	190.00*	152.00*	179.00*	167.00*	188.00$^{\#}$
	Carot.	2.50^{ns}	2.30^{ns}	2.10^{ns}	1.90^{ns}	2.10^{ns}	2.00^{ns}	2.15$^{\#}$
Mean	Chl	648.45	630.45$^{@}$	603.84$^{@}$	574.95$^{@}$	595.64$^{@}$	586.27$^{@}$	
	Carot.	4.01	3.80$^{@}$	3.58$^{@}$	3.25$^{@}$	3.45$^{@}$	3.39$^{@}$	
LSD at 5%	Chl	Dust	1.251	Nematode inoculation	1.694	Interaction	4.148	
	Chl.b	Dust	0.111	Nematode inoculation	0.151	Interaction	NS	

* = data significant with 0 inoculation level and 0% dust concentration at *P*=0.05, ns = Not significant, # = data significant within a column at *P* = 0.05, @ = data significant in a row at *P* = 0.05.

As per results of growth and leaf pigments for the nematode infected tomato plants, they increased upto 30% sawdust additions in the field soil. Promoted plant growth upto 30% dust could probably generate surplus nutrients. On the other hand sawdust amendments up to 30% facilitate the free movement of juvenile (O'Bannon and Raynolds, 1961) through improving the porosity of the field soil which might be responsible for greater root penetration. Greater number of engrossed second stage juvenile (J_2) would subsequently be metamorphosed into greater number of third and fourth stage juveniles (J_3+J_4). They would have to be transformed into females and subsequently through sedentary parasitism.

Table 7. Effect of sawdust on number of J_2 and J_3+J_4 of root-knot nematode on tomato plants.

Sawdust concentration (%)	Parameters	Inoculation levels						
		0	1000	2000	3000	4000	5000	Mean
0	J_2	0.0	1702.0*	1796.0*	1997.0*	1845.0*	1825.0*	1527.50
	J_3+J_4	0.0	390.0*	439.0*	500.0*	478.0*	467.0*	379.0
10	J_2	0.0^{ns}	1772.0*	1836.0*	2005.0*	1865.0*	1835.0*	1552.1^{ns}
	J_3+J_4	0.0^{ns}	415.0*	467.0*	543.0*	538.0*	527.0*	415.0$^{\#}$
20	J_2	0.0^{ns}	1820.0*	1883.0*	2015.0*	1904.0*	1887.0*	1584.8$^{\#}$
	J_3+J_4	0.0^{ns}	445.0*	498.0*	570.0*	553.0*	543.0*	434.8$^{\#}$
30	J_2	0.0^{ns}	1865.0*	1940.0*	2035.0*	2015.0*	1973.0*	1638.0$^{\#}$
	J_3+J_4	0.0^{ns}	470.0*	537.0*	610.0*	594.0*	587.0*	466.3$^{\#}$
40	J_2	0.0^{ns}	1753.0*	1822.0*	1905.0*	1850.0*	1839.0*	1528.2^{ns}
	J_3+J_4	0.0^{ns}	412.0*	440.0*	478.0*	468.0*	455.0*	375.5$^{\#}$
50	J_2	0.0^{ns}	973.0*	1033.0*	1130.8*	1070.0*	1028.0*	872.5$^{\#}$
	J_3+J_4	0.0^{ns}	305.0*	338.0*	369.0*	358.0*	345.0*	285.8$^{\#}$
60	J_2	0.0^{ns}	437.0*	527.0*	638.0*	569.0*	556.0*	454.5$^{\#}$
	J_3+J_4	0.0^{ns}	210.0*	227.0*	265.0*	252.0*	240.0*	199.0$^{\#}$
70	J_2	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0$^{\#}$
	J_3+J_4	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0$^{\#}$
80	J_2	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0$^{\#}$
	J_3+J_4	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0$^{\#}$
90	J_2	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0$^{\#}$
	J_3+J_4	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0$^{\#}$
100	J_2	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0$^{\#}$
	J_3+J_4	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0$^{\#}$
Mean	J_2	0.00	938.4$^{@}$	985.2$^{@}$	1056.2$^{@}$	1008.9$^{@}$	996.6$^{@}$	
	J_3+J_4	0.00	240.6$^{@}$	267.8$^{@}$	303.2$^{@}$	294.6$^{@}$	287.6$^{@}$	
LSD at 5%	J_2	Dust	31.441	Nematode inoculation	42.571	Interaction	104.278	
	J_3+J_4	Dust	16.051	Nematode inoculation	21.734	Interaction	53.236	

* = data significant with 0 inoculation level and 0% dust concentration at P=0.05; ns = Not significant; # = data significant within a column at P = 0.05; @ = data significant in a row at P = 0.05.

The above said reason may be stemmed as a link towards increase in the number of all type of juveniles and galls upto 30% sawdust levels. But egg masses and fecundity showed gradual suppressions with respect to progressive increase in sawdust. They (including juveniles and soils) were absolutely absent up till 70% dust addition treatments.

The inhibitory effect of sawdust on nematodes can be attributed to the formation of phenolic compounds by the decomposition of sawdust (Kokalis-Burelle et al., 1994). For juveniles and galling, the concentration of some formed phenollic compounds of about 30% dust amendments could not have crossed the threshold limit so as

Table 8. Effect of sawdust on number of galls of root-knot nematode on tomato plants.

Sawdust concentration (%)	Inoculation levels						
	0	1000	2000	3000	4000	5000	Mean
0	0.0	75.00*	87.00*	104.00*	96.00*	91.00*	75.50
10	0.0^{ns}	79.00*	95.00*	112.00*	107.00*	102.00*	82.50$^{#}$
20	0.0^{ns}	85.00*	106.00*	118.00*	115.00*	110.00*	89.00$^{#}$
30	0.0^{ns}	93.00*	110.00*	127.00*	120.00*	119.00*	94.83$^{#}$
40	0.0^{ns}	72.00*	85.00*	96.00*	92.00*	88.00*	72.17$^{#}$
50	0.0^{ns}	48.00*	57.00*	66.00*	63.00*	60.00*	49.00$^{#}$
60	0.0^{ns}	27.00*	34.00*	42.00*	40.00*	38.00*	30.17$^{#}$
70	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0$^{#}$
80	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0$^{#}$
90	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0$^{#}$
100	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0$^{#}$
Mean	0.00	43.55$^{@}$	52.18$^{@}$	60.45$^{@}$	57.45$^{@}$	55.27$^{@}$	
LSD at 5%	Dust	1.821	Nematode inoculation	2.468	Interaction	6.047	

Table 9. Effect of sawdust on number of egg mass of root-knot nematode on tomato plants.

Sawdust concentration (%)	Inoculation levels						
	0	1000	2000	3000	4000	5000	Mean
0	0.0	37.0*	48.0*	60.0*	57.0*	52.0*	42.33
10	0.0^{ns}	26.4*	38.0*	47.0*	43.0*	41.0*	32.6$^{#}$
20	0.0^{ns}	24.0*	28.0*	41.0*	36.0*	34.0*	27.2$^{#}$
30	0.0^{ns}	19.0*	25.0*	36.0*	34.0*	28.0*	23.7$^{#}$
40	0.0^{ns}	13.0*	18.0*	33.0*	28.0*	25.0*	19.5$^{#}$
50	0.0^{ns}	7.0*	13.0*	23.0*	19.0*	15.0*	12.8$^{#}$
60	0.0^{ns}	4.0*	7.0*	15.0*	13.0*	8.0*	7.8$^{#}$
70	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0$^{#}$
80	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0$^{#}$
90	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0$^{#}$
100	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0$^{#}$
Mean	0.00	11.9$^{@}$	16.1$^{@}$	23.2$^{@}$	20.9$^{@}$	20.4$^{@}$	
LSD at 5%	Dust	1.191	Nematode inoculation	1.612	Interaction	3.949	

* = data significant with 0 inoculation level & 0% dust concentration at P=0.05; ns = Not significant; # = data significant within a column at P = 0.05; @ = data significant in a row at P = 0.05.

Table 10. Effect of sawdust on fecundity of root-knot nematode on tomato plants.

Sawdust concentration	Inoculation levels						
	0	**1000**	**2000**	**3000**	**4000**	**5000**	**Mean**
0	0.0	405.0*	417.2*	432.0*	428.4*	424.0*	351.10
10	0.0^{ns}	385.2*	394.0*	420.2*	404.6*	392.2*	332.7$^{#}$
20	0.0^{ns}	375.8*	307.6*	398.0*	386.0*	378.0*	307.6$^{#}$
30	0.0^{ns}	366.0*	378.4*	389.0*	376.0*	370.2*	313.3$^{#}$
40	0.0^{ns}	358.4*	367.2*	377.6*	370.0*	365.6*	306.5$^{#}$
50	0.0^{ns}	348.0*	357.4*	368.0*	362.6*	358.0*	299.0$^{#}$
60	0.0^{ns}	339.2*	348.2*	359.5*	352.4*	346.4*	291.0$^{#}$
70	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.0$^{#}$

Table 10. Contd

80	0.0 ns	0.0 ns	0.0 ns	0.0 ns	0.0 ns	0.0 ns	0.0 #
90	0.0 ns	0.0 ns	0.0 ns	0.0 ns	0.0 ns	0.0 ns	0.0 #
100	0.0 ns	0.0 ns	0.0 ns	0.0 ns	0.0 ns	0.0 ns	0.0 #
Mean	0.00	234.3 @	233.6 @	249.5 @	243.6 @	239.5@	
LSD at 5%	Dust	1.690	Nematode inoculation	2.289	Interaction	5.570	

* = data significant with 0 inoculation level and 0% dust concentration at *P*=0.05; ns = Not significant; # = data significant within a column at *P* = 0.05; @ = data significant in a row at *P* = 0.05.

to become toxic for them. However, the reverse happens with egg masses and fecundity even at the same sawdust levels which tend to remain in direct physical contact with the surrounding dust stressed environment. Other cause of their reduction was that of the development and colonization of nematode natural enemies (Oka, 2010; Thoden et al., 2011) in higher sawdust amendment soil. Increased colonization and reproduction of the nematophagous fungi was reported in sawdust amended soil (Hassan et al., 2010).

From the above discussion, we can conclude that sawdust proved detrimental to overall root-knot disease of tomato plants. However, this dust improved growth and leaf pigments was about 30%.

Conflict of Interests

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

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Vol. 13(41), pp. 4081-4092, 8 October, 2014
DOI: 10.5897/AJB2014.13892
Article Number: 497584247976
ISSN 1684-5315
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http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Interaction between faba bean cultivars and the *Rhizobium leguminosarum* strains: Symbiotic N_2 fixation and protein profiles under salt stress

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Received 30 April, 2014; Accepted 29 September, 2014

The efficiency of eight *Rhizobium leguminosarum* strains isolated from root nodules of Faba bean plants which were collected from different Governorate in Egypt has been examined. After inoculation of faba bean seeds with each *Rhizobium* strain individually, we measured the growth promotion, nodule formation and plant protein profile under different concentration of sodium chloride salt stress. The interactions between salt tolerant and sensitive faba bean cultivars and *Rhizobium* strains under NaCl stress were found to be significant. Nitrogen fixation efficiency varied among the eight *Rhizobium* strains. The protein profile using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for both faba bean cultivars revealed that *Rhizobium* strains numbers RL1, 2, 4 and RL 8 showed high level of salt tolerance under 40 and 80 mM NaCl in both faba bean cultivars. *Rhizobium* strains improved the salt tolerance, as is clearly observed in number of nodules and the amount of N_2 fixation. It is known that stress affects the growth, metabolic activity and symbiotic efficiency of *Rhizobium* with faba bean plant but in this study we found that *Rhizobium* strains numbers RL2 and RL 3 showed high efficiency of N_2 fixation and growth promotion in both faba bean cultivar.

Key words: Faba bean, *Rhizobium*, nodulation efficiency, salt stress, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein profile.

INTRODUCTION

Symbiotic nitrogen fixation (SNF) by legumes plays an important role in reinforcing crop productivity and conserving the fertility of peripheral land and in the small-holder system of the semi-arid tropics. It is expected that the importance of legumes and symbiotic nitrogen fixation will continue to increase the development of national

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sus-tainable agriculture. The major step toward maximum SNF technology is the increment of land area under legumes and enhances their grains and fodder yield through over-coming environmental condition problems which limit SNF and legume productivity. However, SNF by legumes is particularly sensitive to environmental stress like drought, soil salinity, acidity and low phosphorus (Zahran, 1999). The most serious threat faced by agriculture in arid and semi-arid regions is salinity (Rao and Sharma, 1995); unsuccessful symbiosis under salt-stress may be due to failure in the infection process, because of the effect of salinity on the establishment of rhizobia (Fauvart and Michiels, 2008). The salt damage on the symbiotic inter-action not only inhibits the formation of nodules, but also leads to the reduction of the growth of the host plant.

Other effects of salinity on the nodulation include forma-tion of non-functional nodules with abnormal structure and degradation of peribacteroid membrane (Bolaños et al., 2003). Strategies to improve legume production in saline environments include selection of host genotypes that are tolerant to high salt conditions; inoculation with salt-tole-rant strains of rhizobia could constitute another approach to improve legume productivity under symbiosis (Hassan and Eissa, 2013). The improving of the symbiotic efficiency and legume production under this constraint should target both symbiotic partners, together with appropriate crop and soil management (Fernandez et al., 2010; Sharma et al., 2013). Furthermore, Nearly 40% of the world land surface can be categorized as having potential salinity problems affecting soil fertility. Most of these areas are confined to the tropics and the Mediterranean regions. It is important to select more effective strains for specific cultivars from the region where common beans are cultivated inten-sively. Moreover, O'Hara et al. (2002) reported that the abundance of diversity in the soil populations of rhizobial strains provides a large resource of natural germplasm to screen desired characteristics present in the natural pool. Also, Workalemahu (2009) stated that the presence of diversity among the strains revealed the possibility of get-ting potentially effective adaptable *Rhizobium* strains that enhance faba bean productivity.

Little is known about salinity effect on nodule pattern distribution in different regions of the root system, and their efficacy to establish a symbiotic association that is, the ability of the *Rhizobium*-legume to develop a partnership for forming nodules. Although the root nodule colonization of *Rhizobium* bacteria are more salt tolerant than their legume hosts, they showed marked variation in salt tolerance. The present study examines the nodulation and nitrogen fixation efficiency of new *Rhizobium* strains under salt stress in faba bean plant to improve the crop productivity and sustaining the fertility of land in arid and semiarid regions.

MATERIALS AND METHODS

Rhizobium leguminosarum strains

Eight *Rhizobium* strains were isolated from faba bean root nodules that were collected from different locations of Egyptian soil. These strains of *R. leguminosarum* were identified according to Shoukry et al. (2013). All strains were purified, characterized and identified as belonging to the genus *R. leguminosarum* bio*. viciae*. The purified strains have been tested for their nodulation and growth potential under salt conditions. Faba bean seeds were inoculated with *Rhizobium* strains to study their efficiency of nodulation by measuring number of nodule; plant height; plant fresh and dry weight; total nitrogen; phosphorus content; potassium and sodium content in faba bean plants under different concentration of sodium chloride NaCl (0; 10; 20; 40 and 80 mM) to be added in irrigation water.

Growth conditions of the *Rhizobium* strains and experimental pots

The selected strains were examined to grow in Yeast extract Mannitol (YEM) broth (Vincent, 1970) containing 0 to 80 mM NaCl (pH7.0) with three replicates in a gyratory shaker at 150 rpm and 30°C. Cells grown in YEM brothfor 18 h were used as an inoculums (10^9 C.F.U. /ml) and then sown in 42X60 cm pots (in three replicates), each containing 5 kg sandy soil. The soil in these pots was saturated by watering with saline water (1000 ml of saline water perpot) containing 0 to 80 mM NaCl. Inoculated plants were also maintained on water without NaCl as controls. The plants were irrigated with 300 ml Brogan's modified cronies solution which consists of g/L: KCl_2 10.09; $CaSO_4$-$2H_2O$ 2.5; $MgSO_4$-H_2O 2.5; Ca (PO_4) 2 2.5 and Fe PO_4 2.5 (Allen, 1961) nitrogen free with different concentration of NaCl: 0; 10; 20; 40 and 80 mM /pot/three days. The plants were removed after 60 days then number of nodules, plant height (cm), fresh weight (g) and dry weight (g) were recorded.

Salt stress

Tolerant and salt sensitive cultivars seeds of faba bean (Giza 843 and Sakha 1 cultivars) were used in this study obtained from Legumes Research Department Agronomy Research Institute, Agricultural Research Center, Giza, Egypt. The pot experiment was carried out at experimental farm Faculty of Agriculture Al- Azhar University, Nasr city, Cairo, Egypt.

N, P, K and Na determination

The plant samples were collected from each treatment randomly. Fresh and dry biomass of whole plant was recorded. Total nitrogen, sodium, phosphorus and potassium contents of treated faba bean plants were estimated in the plant digest according to the method described by Faithfull (2002).

SDS-protein electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli, (1970). Total soluble proteins of the whole plant powder fully were taken from two cultivars, Sakha1 and Giza823 after treatment with NaCl in large pot. Protein fractionations were performing exclusively on vertical slab (19.8 × 26.8 × 0.2 cm) gel using the electrophoresis apparatus manufactured by LABOCONCO. Gels were analyzing using Total Lab TL100.

Statistical analysis

The data were subjected to statistical analysis, employing F-test for

significance ($P \leq 0.05$) and computing of "Least Significant Difference (L.S.D.)" values to separate means in different statistical groups as described by Gomez and Gomez (1984).

RESULTS AND DISCUSSION

Number of nodules/plant

There was a significant difference ($P \leq 0.05$) in the number of nodules per plant between different *Rhizobium* strains. However, data in Tables 1 and 2 showed that the strain RL1 revealed the highest values for number of nodules above all strains under different concentrations of NaCl in the two faba bean cultivars (Giza 843 and Sakha 1). The average number of nodules for RL1 strain was 198.37 nodules per plant at control treatment and decreased to 62.59 nodules per plant at 80 mM NaCl concentration in Giza 843 cultivar while, RL1 strain had 175 nodules per plant at control treatment and 36.33 nodules per plant at 80 mM NaCl concentration in Shaka1 cultivar. The negative effect of a high level of salinity on number of nodules per plant were also found by Fetyan and Mansour (2012), who reported that high salt concentration inhibited the growth of the three *Rhizobium* strains. Furthermore, Younesi et al. (2013) reported that nodulation was completely inhibited under salt stress condition. In this respect, Fahmi et al. (2011) reported that the increase of seawater concentration (salinity level) decreased the average number of nodule through nodule formation by the inhibition of initial steps of *Rhizobium*-legume symbioses. In this concern, the variation in nodulation could be due to low rhizobial density, incompatibility of the rhizobia and edaphic factors that hinder the effecttiveness of the rhizobia. Moreover, Fetyan and Mansour (2012) stated that the limitation of symbiosis under saline conditions may be due to: (i) survival and proliferation of *Rhizobium* spp. in the soil and rhizosphere; (ii) inhibiting the infection process, (iii) directly affecting root nodule function, or (iv) reducing plant growth, photosynthesis, and demand for nitrogen.

On other hand, Belal et al. (2013) showed that the *R. leguminosarum* bio*. viciae* isolated from different geographic regions soils are able to survive, grow and effecttively nodulated on faba bean even at high salt concentrations.

Plant height (cm)

Significant differences ($P \leq 0.05$) were observed between eight *Rhizobium* strains under different salt concentrations on plant height (Tables 1 and 2). The results reveal that the tallest plants (63.56 and 61.22 cm) were obtained in plants inoculated with RL6 and RL7 strains under control conditions; the same two strains had given the tallest plants (50 and 46.63 cm) under 80 mM NaCl concentration in Shaka1 cultivar. Conversely, the tallest plants (68 and 66.67 cm) were obtained in plants treated with RL5 and RL4 strains under 0 mM NaCl (control) while, under 80 mM NaCl concentration the tallest plants (56.49 and 56 cm) were obtained in plants treated with RL1 and RL8 strains in Giza 843. The variation among treated plants under salt stress and control in plant height may be due to the harmful effects of salinity on availability of nutrients for growth of plants. Supporting results were obtained by Al-Shaharani and Shetta (2011) who indicated that salt stress caused a significant depression in seedling growth parameters (seedling height) of both studied *Acacia* species and it seemed to reduce the availability of the nutrients required for the growth and then development of the plants comparing with the control.

Fresh weight (g/plant)

Fresh weights of faba bean plants treated with different *Rhizobium* strains were significantly affected at the 5% level. However, careful observations of data in Tables 1 and 2 clearly show that plants inoculated with RL7 and RL2 strains were superior in fresh weight per plant under all NaCl concentrations in Giza 843 and had 185.55, 183.67, 145 and 108.33 g/plant under control and 80 mM NaCl respectively. On contrast, the maximum amounts of fresh weight per plant (164.54 and 159.07 g/plant) were recorded in plants inoculated with RL4 and RL2 strains, respectively, under control condition. While the maximum amounts of fresh weight per plant (96.22 and 86.33 g/plant) were recorded in plants treated with RL5 and RL6 strains respectively under 80 mM NaCl. This obtained result goes in line with those findings by Alshammary et al. (2004) who reported that salinity reduces shoot and root weights in several legumes. In addition, increasing the salinity level of solution reduced the fresh and dry weights of Savory plant. However, Öğütçü et al. (2010) concluded that salinity levels negatively affected all parameters of growth such as root and shoot growth in chickpea plants. The decrease in plant biomass production due to salinity may be attributed to low or medium water potential, specific ion toxicity, or ion imbalance.

Dry Weight (g/plant)

There were significant differences ($P \leq 0.05$) in the dry weight between different *Rhizobium* strains as shown in Tables (1 and 2). However, the maximum amounts of dry weight per plant (28.67 and 27.51 g/plant) were recorded in plants inoculated with RL2 strain under 20 mM of NaCl and plants inoculated with RL7 strain under control respectively without significant differences between them. Moreover, plants inoculated with RL7, RL6 and RL2 strains gave the maximum amounts of dry weight per plant (15.85, 15.48 and 14.67 g/plant) with significant differences between them and control in Giza 843 cultivar. Regarding Sakha 1, the maximum amounts of

Table 1. Effect of salinity on number of nodules, plant height, fresh and dry weights of faba bean cultivar Giza 843 inoculated with different *Rhizobium* strains.

***Rhizobium* strains**	**Salinity levels (mM)**				
	0	**10**	**20**	**40**	**80**
Number of Nodules / Plant					
RL1	198.37	157.67	127.68	114.70	62.59
RL2	126.77	116.18	125.00	76.33	35.67
RL3	41.33	38.67	33.33	22.67	11.78
RL4	69.67	68.67	57.33	41.67	27.22
RL5	93.21	81.33	79.11	55.66	25.67
RL6	137.67	136.33	109.00	67.67	39.67
RL7	135.33	127.67	93.67	61.48	27.67
RL8	146.37	158.33	96.65	79.52	64.67
LSD at 5% = 8.13					
Plant Height (cm)					
RL1	54.89	54.62	49.83	43.16	56.49
RL2	55.33	53.34	54.21	54.07	48.67
RL3	56.21	52.33	50.67	47.33	46.00
RL4	66.67	65.67	51.33	48.22	45.33
RL5	68.00	58.67	47.33	46.11	44.67
RL6	59.71	52.33	52.47	48.67	44.67
RL7	59.85	56.00	53.67	51.00	46.00
RL8	49.81	47.67	49.00	42.00	56.00
LSD at 5% = 7.99					
Fresh Weight (g/plant)					
RL1	129.87	119.04	103.10	92.67	63.48
RL2	183.67	179.00	152.00	133.00	108.33
RL3	127.00	82.11	81.33	68.67	63.00
RL4	179.33	176.00	164.33	104.11	98.53
RL5	179.22	165.89	156.33	123.43	108.52
RL6	163.52	158.00	140.00	120.00	106.00
RL7	185.55	178.00	163.00	160.00	145.00
RL8	148.25	146.00	143.00	134.00	91.00
LSD at 5% = 7.89					
Dry Weight (g/plant)					
RL1	17.88	17.88	14.15	11.27	9.55
RL2	27.33	28.67	23.33	18.33	14.67
RL3	18.92	13.56	11.54	9.33	6.63
RL4	26.12	22.55	18.95	16.74	14.52
RL5	19.55	18.95	16.18	15.77	14.18
RL6	18.92	17.87	16.77	15.49	15.48
RL7	27.51	27.21	24.32	19.89	15.85
RL8	18.67	15.00	13.22	12.00	9.85
LSD at 5% = 4.34					

dry weight per plant (24.48 and 22.03 g/plant) were recorded in plants inoculated with RL2 and RL4 strains under control. Furthermore, plants inoculated with RL5, RL6 and RL7 strains gave the maximum amounts of dry weight per plant (12.16, 10.22 and 10.04 g/plant) with significant differences between them and control. Peoples

Table 2. Effect of salinity on number of nodules, plant height, fresh and dry weights of faba bean cultivar Shaka 1 inoculated with different *Rhizobium* strains.

***Rhizobium* strains**	**Salinity Levels (mM)**				
	0	**10**	**20**	**40**	**80**
Number of nodules / Plant					
RL1	175.00	135.00	113.00	78.33	36.33
RL2	115.41	76.53	63.34	61.33	27.74
RL3	21.78	19.17	10.32	7.89	5.10
RL4	66.55	62.93	37.57	28.94	17.20
RL5	78.62	69.33	59.33	50.40	23.22
RL6	114.91	107.40	97.33	38.67	27.00
RL7	107.33	66.67	55.33	34.33	19.67
RL8	77.56	81.00	41.33	38.00	25.00
LSD at 5% = 5.97					
Plant height (cm)					
RL1	59.00	53.67	53.33	45.33	40.33
RL2	57.85	56.22	53.26	46.88	43.37
RL3	54.91	53.49	42.03	41.18	40.29
RL4	59.87	56.88	51.03	43.62	38.15
RL5	49.52	48.00	46.37	44.66	41.33
RL6	63.56	59.65	50.67	48.33	46.63
RL7	61.22	53.33	59.00	51.00	50.00
RL8	56.33	50.60	55.32	52.68	45.00
LSD at 5% = 7.03					
Fresh weight (g/plant)					
RL1	85.33	76.00	76.33	45.67	28.00
RL2	159.07	153.40	114.07	100.21	85.01
RL3	95.85	74.59	66.41	60.41	62.47
RL4	164.54	162.51	151.16	98.63	77.77
RL5	152.19	144.89	126.33	109.55	96.22
RL6	139.00	125.00	115.00	109.00	86.33
RL7	125.67	127.00	113.07	102.02	76.00
RL8	108.52	100.00	90.00	83.00	76.00
LSD at 5% = 7.85					
Dry weight (g/plant)					
RL1	16.85	14.11	11.27	9.62	7.26
RL2	24.48	16.57	12.29	11.91	9.52
RL3	17.92	8.59	7.38	7.70	6.78
RL4	22.03	20.20	15.81	11.69	8.74
RL5	17.17	16.51	15.94	12.51	10.22
RL6	14.52	16.52	13.87	13.57	12.16
RL7	15.96	14.23	13.54	11.23	10.04
RL8	15.55	14.37	12.32	9.62	5.95
LSD at 5% = 3.82					

et al. (2002) explained that shoot dry matter is a good indicator of relative isolate effectiveness. However, Al-Shaharani and Shetta (2011) indicated that salt stress caused a significant depression in seedling growth parameters (dry weight) in both *Acacia* species it seemed to reduce the availability of the nutrients required for the growth

and then development of the plants comparing with the control. Belal et al. (2013) found that *R. leguminosarum* bv *viciae* improved the growth parameters dry weight of shoot of faba bean plants while, Mnalku et al. (2009) found that significant correlation between nodule number and nodule dry weight with shoot dry weight substantiates the N fixation efficiency of strains (nodules).

Nitrogen percentage

Nitrogen is an essential and often limiting plant nutrient in crop production. Data tabulated in Tables 3 and 4 showed that, significant differences at the 5% level among different applied *Rhizobium* strains. However, total nitrogen content accumulated in faba bean plants inoculated with RL3, RL4 and RL1 strains had showed more nitrogen content above all strains at different NaCl concentrations in Giza 843 while, faba bean plants inoculated with RL5, RL2 and RL8 strains had showed more nitrogen content above all strains at different NaCl concentrations in Shaka1 cultivar. The maximum nitrogen percentage (4.09%) were noticed in plants treated with RL3 strain in Giza 843 cultivar at 80 mM NaCl followed by plants treated with RL5 strain which had given 4.92% at 40 mM NaCl in Shaka1 cultivar. These results are in agreement with those obtained by Al-Fredan (2006) indicate that locally isolated strains of *R. leguminosarurn* inoculated faba bean plants fixes much greater N, than when introduced strain are used. Also, salinity tolerant rhizobial strains increased significantly the total N2 mg/plant in saline sandy soil compared with the inoculation with salinity un-tolerant rhizobial isolates. However, the harmful effects of salinity on Nitrogen fixing or content were obtained by many authors (Zahran, 2001; Zheng et al., 2009; Al-Shaharani and Shetta, 2011). The reasons for decline nitrogen content under salt stress that chlorine ions limit the absorption of N_2. Antagonistic effect between Cl and N_2 is well known in glycophytes than halophytes plants; the latter are able to absorb efficiency N_2-even under high salt condition. Moreover, salt-induced distortion in nodule structure could also be a reason for the decline in the N_2 fixation rate by legumes subjected to salt stress. In addition, the reduction of N_2-fixing activity by salt stress usually attributed to a reduction in respiration of the nodules (Kenenil et al. 2010).

Phosphorus percentage

Overall results show significant differences at the 5% level among different *Rhizobium* strains applied. However, data in Tables 3 and 4 revealed that, the highest percentages of phosphorus attained in faba bean plants inoculated with RL3 and RL2 strains were 0.30 and 0.21% at 40 mM NaCl and control treatments respectively, in Giza 843 cultiver whereas, the highest percentages of phosphorus attained in faba bean plants inoculated with RL5, RL2 and RL3 strains were 0.25, 0.23 and 0.23% at 80, 20 and 40 mM NaCl treatments respectively, in Shaka1 cultivar. Salt stress markedly decreased the plant P content in both alfalfa cultivars. Therefore, it can be stated that Salinity may be limiting factor for efficient nodulation and nitrogen fixation by decreasing the P content in the plants (Younesi et al. 2013).

Potassium percentage

Potassium percentage differed significantly ($P \leq 0.05$) between various treated faba bean plants with eight *Rhizobium* strains. However, data in Tables 3 and 4 showed that, the *Rhizobium* strains RL1 and RL2 are superior strains that had given maximum potassium percentages at different salinity levels in Giza 843 and Shaka1 cultivars respectively. Moreover, maximum potassium percentages 3.09 and 2.80 were recorded in plants inoculated with RL1 and RL5 at 80 and 20 mM NaCl in Giza 843 and Shaka1 cultivars respectively. These results were in line with those findings by Younesi et al. (2013) who reported that applying bacterial treatment, especially inoculation by *Rhizobium* strains were significantly increased the K^+ content of both alfalfa cultivars under salt stress conditions. Increased K concentration under saline conditions may help to decrease Na uptake and this can indirectly maintain the growth of the plant (Giri and Mukerji, 2004).

Sodium percentage

Results in Tables 3 and 4 indicate that, treated faba bean plants with different *Rhizobium* strains had effect on sodium percentage. However, careful observations of data clearly showed that, the highest sodium percentages 0.57, 0.52 and 0.51% were recorded in plants treated with RL3, RL1 and RL5 at control, 20 and 80 mM NaCl treatments in Giza 843cultivar. Conversely, the highest sodium percentages 0.59, 0.53 and 0.52% were recorded in plants treated with RL6, RL2 and RL8 at control, 80 and 40 mM NaCl treatments in Shaka1 cultivar. The *Rhizobium* and *Pseudomonas* can modify salt stress in alfalfa by increasing the total uptake of K, P, N and decreasing Na+ accumulation as compared to control. Also, accumulation of Na was strongly influenced by storage of other cations, particularly K (Younesi et al., 2013).

Faba bean Vegetative storage protein under salt stress inoculated with different strains of *Rhizobium*

The interaction between eight different *Rhizobium* strains with two faba bean varieties sensitive (Sakha1) and tolerant to salt stress (Giza 843) under different concentration of NaCl (10, 20, 40, 80 mM) was investigated using SDS-PAGE as illustrated in Figure 1. The analyzed vegetative storage protein profile indicates the nitrogen fixation

Table 3. Effect of salinity levels on nitrogen, phosphorus, potassium and sodium percentages of faba bean cultivar Giza 843 inoculated with different *Rhizobium* strains.

***Rhizobium* strains**	**Salinity Levels (mM)**				
	10	**20**	**40**	**80**	**Mean**
Nitrogen percentage					
RL1	1.02	1.44	1.44	1.53	1.36
RL2	1.50	1.34	1.21	1.02	1.27
RL3	1.09	1.47	1.85	4.09	2.13
RL4	1.53	1.12	1.66	1.21	1.38
RL5	1.02	1.09	1.31	1.02	1.11
RL6	1.12	1.44	1.21	1.44	1.30
RL7	1.40	1.15	1.21	1.40	1.29
RL8	1.28	1.37	1.12	1.15	1.23
Mean	1.25	1.30	1.38	1.61	
Phosphorus percentage					
RL1	0.10	0.03	0.07	0.14	0.09
RL2	0.21	0.16	0.01	0.07	0.11
RL3	0.07	0.14	0.30	0.12	0.16
RL4	0.13	0.03	0.05	0.14	0.09
RL5	0.09	0.05	0.11	0.03	0.07
RL6	0.13	0.08	0.04	0.08	0.08
RL7	0.15	0.05	0.07	0.11	0.10
RL8	0.07	0.08	0.05	0.11	0.08
Mean	**0.12**	**0.08**	**0.09**	**0.10**	
Potassium percentage					
RL1	1.59	1.98	2.15	3.09	2.20
RL2	0.19	1.50	1.05	0.99	0.93
RL3	0.89	1.08	1.08	1.51	1.14
RL4	1.83	0.94	1.22	1.49	1.37
RL5	1.66	1.36	2.07	1.65	1.69
RL6	1.23	1.50	0.85	1.29	1.22
RL7	1.31	1.85	1.41	1.40	1.49
RL8	1.56	1.30	1.62	2.13	1.65
Mean	**1.28**	**1.44**	**1.43**	**1.69**	
Sodium percentage					
RL1	0.37	0.52	0.43	0.33	0.41
RL2	0.21	0.49	0.21	0.30	0.30
RL3	0.57	0.32	0.36	0.31	0.39
RL4	0.43	0.45	0.29	0.46	0.41
RL5	0.37	0.40	0.40	0.51	0.42
RL6	0.22	0.31	0.18	0.43	0.29
RL7	0.30	0.30	0.39	0.30	0.32
RL8	0.41	0.40	0.44	0.43	0.42
Mean	0.36	0.40	0.34	0.38	

efficiency of the Rhizobium strains under salt stress using SDS-PAGE. Our results reveal various responses of *Rhizobium* strains for quantitative and qualitative protein bands in certain plant age and type under NaCl treatment. However, the low concentration of NaCl 10 and 20 mM does not affect the quantitative profile band compared to the control in both faba bean cultivars with all *Rhizobium* strains. Four *Rhizobium* strains (RL4, 5, 7 and 8) showed

Table 4. Effect of salinity levels on nitrogen, phosphorus, potassium and sodium percentages of faba bean cultivar Shaka1 inoculated with different *Rhizobium* strains.

***Rhizobium* strains**	**Salinity Levels (mM)**				
	10	**20**	**40**	**80**	**Mean**
Nitrogen Percentage					
RL1	2.17	1.72	2.46	1.66	2.00
RL2	3.58	1.92	2.78	2.81	2.77
RL3	1.85	1.88	2.30	1.98	2.00
RL4	1.92	2.11	1.44	0.96	1.61
RL5	1.92	2.97	4.92	2.36	3.04
RL6	2.04	2.30	1.76	1.82	1.98
RL7	2.97	2.01	2.07	1.98	2.26
RL8	2.14	2.07	2.81	2.04	2.27
Mean	2.32	2.12	2.57	1.95	
Phosphorus percentage					
RL1	0.15	0.13	0.20	0.18	0.17
RL2	0.23	0.23	0.19	0.18	0.21
RL3	0.11	0.13	0.23	0.21	0.17
RL4	0.13	0.09	0.16	0.08	0.12
RL5	0.13	0.04	0.15	0.25	0.14
RL6	0.05	0.11	0.09	0.04	0.07
RL7	0.15	0.17	0.20	0.19	0.18
RL8	0.14	0.14	0.17	0.12	0.14
Mean	0.14	0.13	0.17	0.16	
Potassium percentage					
RL1	1.45	1.10	1.07	0.75	1.09
RL2	1.56	1.53	1.81	1.54	1.61
RL3	1.31	1.12	1.72	1.73	1.47
RL4	1.56	1.15	1.44	1.54	1.42
RL5	1.71	2.80	0.81	0.84	1.54
RL6	1.75	1.25	1.21	0.92	1.28
RL7	1.18	1.11	1.33	1.06	1.17
RL8	1.61	1.67	1.14	1.06	1.37
Mean	1.52	1.47	1.32	1.18	
Sodium percentage					
RL1	0.38	0.28	0.36	0.32	0.34
RL2	0.35	0.31	0.27	0.54	0.37
RL3	0.27	0.38	0.39	0.36	0.35
RL4	0.34	0.45	0.40	0.36	0.39
RL5	0.36	0.42	0.41	0.28	0.37
RL6	0.59	0.34	0.29	0.27	0.37
RL7	0.39	0.34	0.40	0.31	0.36
RL8	0.42	0.36	0.53	0.39	0.43
Mean	0.39	0.36	0.38	0.35	

salt stress adaptation in both faba bean cultivars. They accumulate more band quantity gradually from 10 to 40 mM NaCl and then dramatically decreased in 80 mM NaCl concentration while, other strains (RL2, 3 and 6) showed increase of bands profile quantity from 10 to 80 mM NaCl. It was obvious to detect some unique bands (29 and

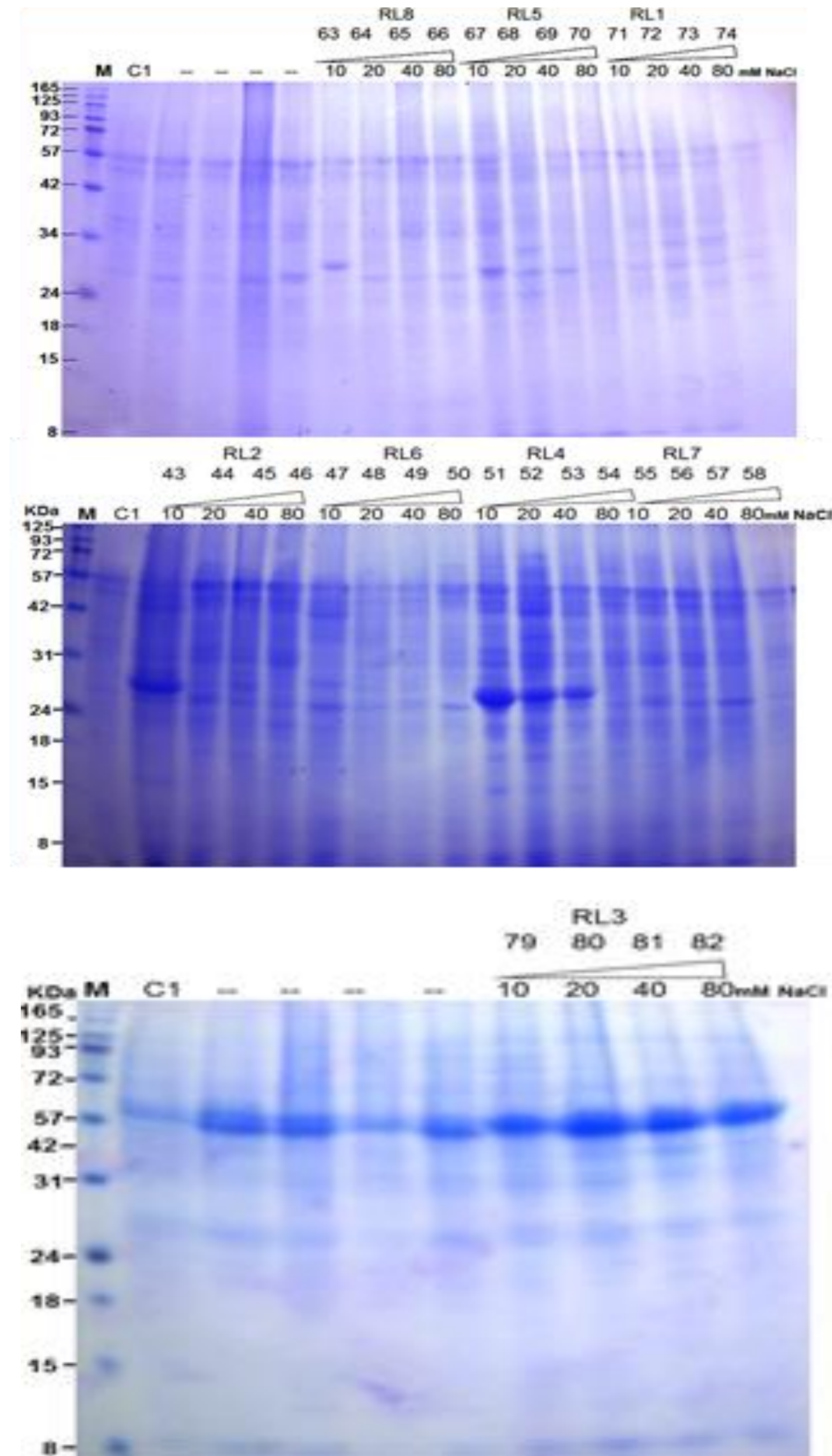

Figure 1. SDS-PAGE soluble protein profile of the interaction between Sakha 1 faba bean cultivar and the eight *Rhizobium* strains (RL 1, 2, 3, 4, 5, 6, 7 and 8) under four different concentration of NaCl (10, 20, 40 and 80 mM).

30 KDa) present only with RL5, 6 and 7 with Giza 843 under 10 mM NaCl and with RL 8 in all NaCl concentrations (Figure 2). Surprisingly the salt sensitive faba bean cultivar. Sakha1 showed the same unique bands (29 and 30 KDa) in most of the *Rhizobium* strains and NaCl concentrations. Our SDS protein profile support

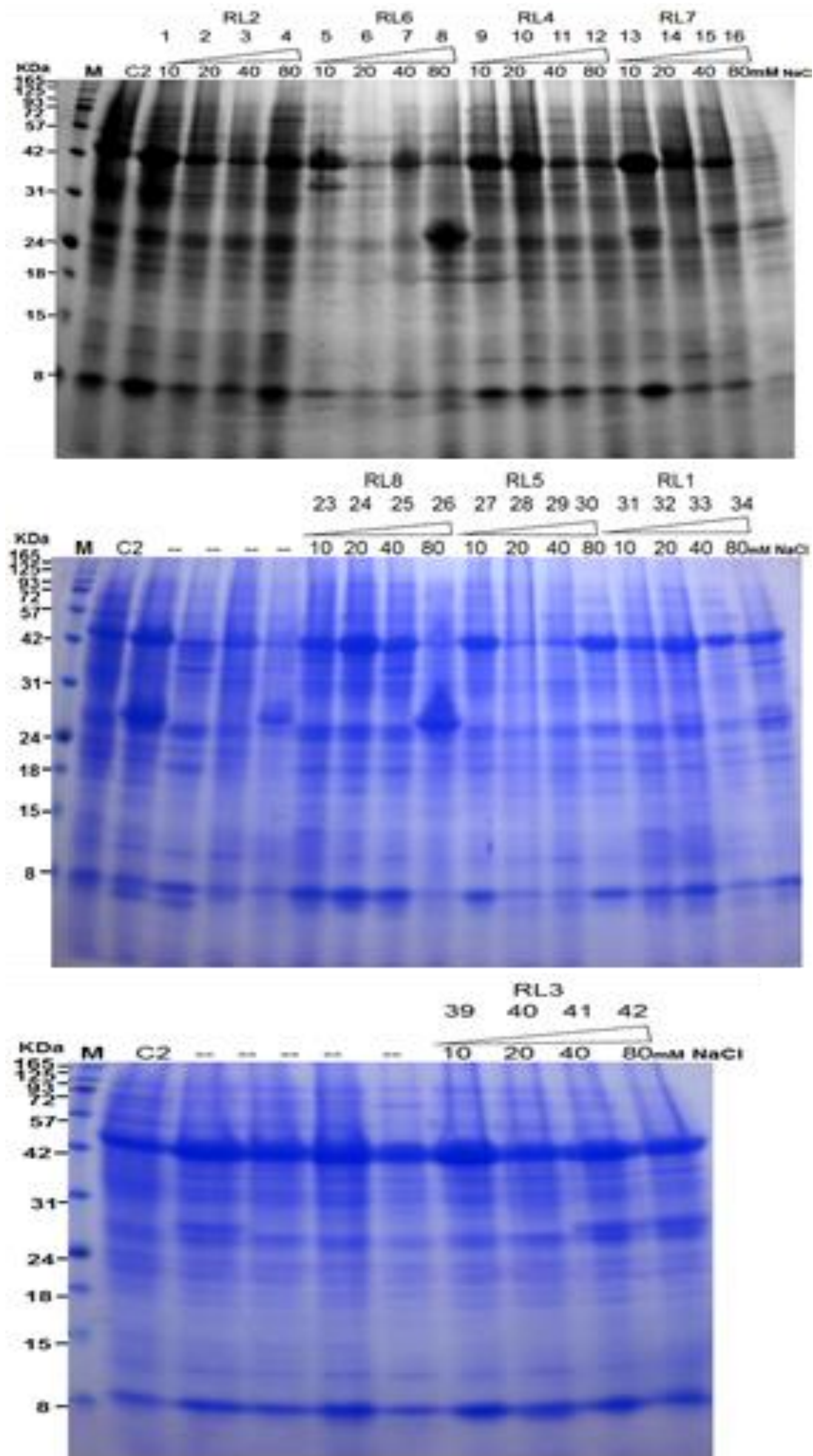

Figure 2. SDS-PAGE soluble protein profile of the interaction between faba bean Giza843 cultivar and the eight *Rhizobium* strains (RL 1, 2, 3, 4, 5, 6, 7 and 8) under four different concentration of NaCl (10, 20, 40 and 80 mM).

the positive effect of the *Rhizobium* strains on faba bean cultivars which showed high phosphor and nitrogen content like RL3, RL4 and RL8 in spite of their quantitative decrease of their fresh, dry weight, plant height and number of nodules

measurements.

RL2 and RL3 interaction protein profile with both faba bean cultivars reveals a very stable and adapted behavior to the NaCl treatment. The decrease in soluble protein content of nodules is a general response to salt stress of many legume plants while, Fahmi, et al. (2011) reported that, the decrease in soluble protein of the nodules may be due to a protein breakdown or to an alteration in the incorporation of amino acids into protein. However, the importance of nutritional value in faba bean is based on its high protein concentration and symbiotically fixed nitrogen with Rhizobia (Mudgal et al., 1997). Few bands (29 and 30 KDa) were observed with the *Rhizobium* strains RL1, 3 and 8 in both faba bean cultivars under high concentrations of NaCl 40 and 80 mM. Zahran (1999) who reported the appearance of new protein bands in SDS-PAGE profiles of rhzobia from woody legumes grown under salt stress had reported similar results.

These two bands based on their molecular size are likely to be nitrogen fixation proteins. The nitrogen fixation regulatory proteins (NIFL) involved in regulation of transcriptional activation of nitrogen fixation and removal radicals contributed in environmental stress like falavoproteins (Heazlewood et al., 2004; Araujo et al., 2010). On the contrary, one approach to understanding the ability of *Rhizobium* to tolerate salt stress has been to identify stress-induced changes of individual proteins under the assumption that stress adaptation results from alterations in gene expression.

Conclusion

In the present study, the interactions between these strains of *R. leguminosarum* and faba bean varied based on several factors like plant genotype and *Rhizobium* strains. Nitrogen fixation response to salt varied between the eight *Rhizobium* strains. Four *Rhizobium* strains (RL1, 2, 4 and 8) showed tolerance under concentrations 40 and 80 mM of NaCl with both faba bean cultivars. The present results, it may be concluded that salt stress affects the growth, metabolic activity and symbiotic efficiency of different strains of *Rhizobium* and faba bean cultivars. However, important variability was observed amongst the sensitive and tolerant faba bean cultivars. An extensive work need to be done to identify these protein bands and analyzed their function in nitrogen fixation under salt stress in Egyptian environment conditions.

Conflict of Interests

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

ACKNOWLEDGEMENTS

We would like to thank the Agricultural Research Center, Giza, Egypt for the supply of seeds.

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Vol. 13(41), pp. 4093-4099, 8 October, 2014
DOI: 10.5897/AJB2014.14079
Article Number: 117151747977
ISSN 1684-5315
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http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Effect of UV-C irradiation on antioxidant activities, total phenolic and flavonoid contents and quantitative determination of bioactive components of *Moringa oleifera* Lam. shoot culture

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Received 30 July, 2014; Accepted 29 September, 2014

Effect of UV-C irradiation on the antioxidant activities of shoot cultures of *Moringa oleifeira* Lam. was investigated. Total phenolic and flavonoid contents and the antioxidant bioactive components were determined. The shoots of *M. oleifeira* were cultured for 6 weeks on Murashige and Skoog (MS) mediums containing 0.5 mg/L 6- benzyladenine (BA) for multiple shoot formation. Multiple shoots were treated with UV-C irradiation for 0 min (for the control group) 5, 10 and 15 min (for the experimental groups). After 4 weeks of culture, the shoots were extracted with methanol and analyzed for antioxidant activities using 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) scavenging and ferric reducing power (FRP) assay and the total phenolic and flavonoid contents were determined. Quantitative analysis of active compounds was accomplished by high performance liquid chromatography (HPLC). The results indicate that the shoots treated with UV-C irradiation for 10 min exhibited the highest antioxidant activities at IC_{50} of 31.43 mg/mL using DPPH scavenging assay, 58.98 mg TEAC/100 g fresh weight (FW) using ABTS scavenging assay and 33.78 mM $FeSO_4$ /100 g FW using FRP assay. The total phenolic and flavonoid contents were 112.56 mg GAE/ 100 g FW and 65.31 mg QE/100 g FW, respectively. Crypto-chlorogenic acid, isoquercetin and astragalin were the highest antioxidant bioactive components with values of 30.10, 61.21 and 12.67 ng/mL, respectively. UV-C irradiation can stimulate the antioxidant capacities of *M. oleifera* shoot cultures. Our study will provide useful knowledge and can be utilized for improving the quality of *M. oleifera* raw materials in herbal supplementary food and medical uses.

Key words: *Moringa oleifeira* Lam., antioxidant activities, UV-C irradition, plant tissue culture.

INTRODUCTION

Moringa oleifera Lam. (Horse radish tree or Drumstick tree) is in the Moringaceae family. It is a plant that originated in Asia, Asia minor and Africa (Mughul et al., 1999). The medicinal properties of it are anti-inflam-

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Figure 1. A 6 week old shoot of *M. oleifera* that had been cultured on MS medium containing 0.5 mg/L BA.

matory, bactericide, anti-diuretic, anti-cancer, reducing blood pressure, relieving joint pain, reducing cardiovascular disease and reducing rheumatism (Anwar et al., 2007; Chumark et al., 2008; Anjula et al., 2011). Besides these it contains some phytochemicals such as group glucosinolates effects against the formation of cancer cells and increasing glutathione. It contains important antioxidants which are phenolic compounds and flavonoids such as rhamnetin, gallic acid, cryptochlorogenic acid, isoquercetin and kaemferol which are high in antioxidants (Bennett et al., 2003; Brahma et al., 2009; Vongsak et al., 2012). The IC_{50} of *M. oleifeira* leaves were extracted by methanol using 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay was 246.06 μg / mL (Maksab and Wichairam, 2009). At present leaves of *M. oleifera* are used as traditional medicines in capsule form. A shortage of young leaves occurred due to insect pests which resulted in the reduction of these traditional medicines.

Some secondary metabolites in plant tissue culture can be stimulated; for example, increasing the amount of isoflavonoid, anthraquinone and anthocyanin production in callus culture (Fedoreyer et al., 2000; Mischenko et al., 1999). The kinds and amount of growth regulators could enhance antioxidant features and total phenolics in plants. The Murashige and Skoog (1962) medium containing 6-benzyladenin (BA) could produce secondary metabolites which have the same chemical composition as the natural plants (Suriyaphan and Matchachip, 2009; Polsak, 2003). The condition of a culture media could be adjusted to incorporate biotic elicitors such as chitosan, chitin and enzymes as well as abiotic elicitors such as oxidative stress as ultraviolet and plant wounded (Benhamou, 1996).

UV-C irradiation (200-280 nm) could increase the activity of defense enzymes and could increase antioxidant activity such as ascorbic acid, anthocyanin synthesis, and total phenolic. It could also help delay senescence in strawberrys (Erkan et al., 2008). There were several reports that UV-C irradiation was used to stimulate the production of antioxidant capacities in the broccoli and Ceylon spinach (Costa et al., 2006; Pumchaosuan and Wongroung, 2008). UV-C irradiation could also stimulate enzyme-associated antioxidant activities including superoxide dismutase, catalase and peroxidase (Erkan et al., 2008). UV-C irradiation at 3.6 KJ/m^2 could inhibit the declining of vitamin C and carotenoids (Burana and Srilaong, 2009). Therefore, this research aimed to stimulate the oxidative stress in shoots of *M. oleifera* by UV-C irradiation in order to promote antioxidant activities, total phenolic and flavonoid contents and antioxidant bioactive components.

MATERIALS AND METHODS

Chemicals

Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium was prepared in-house. 6- benzyladenine (BA), folin-ciocalteaut reagent and phosphate buffered saline pH 7.4 (PBS) were purchased from Sigma-Aldrich, USA. 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), trolox, gallic acid, aluminum chloride, trichloroacetic acid, potassium dihydrogen phosphate and hydrogen peroxide were purchased from Merck (Darmstadt, Germany). Potassium ferricyanide and ferric chloride were obtained from Fluka Biochemika (Steinheim, Germany).

Sodium bicarbonate, and ferrous sulfate were purchased from Ajax Finechem (NSW, Australia). Isoquercetin, crypto-chlorogenic acid and astragalin were purchased from Biopurify China with purity more than 95%. Methanol (HPLC grade) was purchased from RCI Labscan (Thailand). Deionized water was purified from the Ultra clear series TWF (Siemens, Germany). All chemicals and solvents were of analytical grade if not stated otherwise.

Plant materials

M. oleifera plants in this study were collected from a natural source in Uttaradit Province, Thailand. The plant was identified by the author. A voucher specimen was kept at the Science and Technology Center, Uttaradit Rajabhat University, Uttaradit, Thailand.

Plant culture

Shoots of *M. oleifera* were rinsed in sterilized water. Then explants were sterilized by soaking in 15% clorox solution for 7 min and in 10% clorox solution for 7 min. Then they were washed three times with sterile distilled water, cut into pieces of 0.5-1.0 cm in length, and put on the MS medium containing 0.5 mg/L BA (Petchang, 2011) to induce multiple shoot formation (Figure 1). The culture conditions were set at a temperature of 25±3°C and 16 h photoperiod (40 $\mu mol/m^2/s$) by mercury fluorescent lamps for 6 weeks.

UV-C illumination

The UV-C illumination method was modified from Erkan et al.

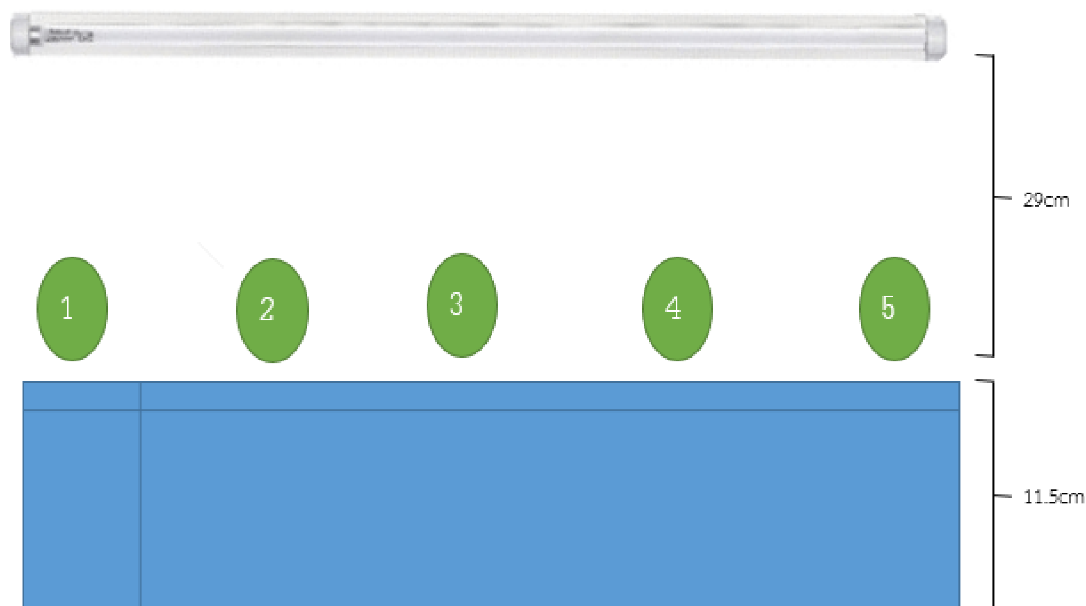

Figure 2. Diagram showing the UV-C treatment of this study.

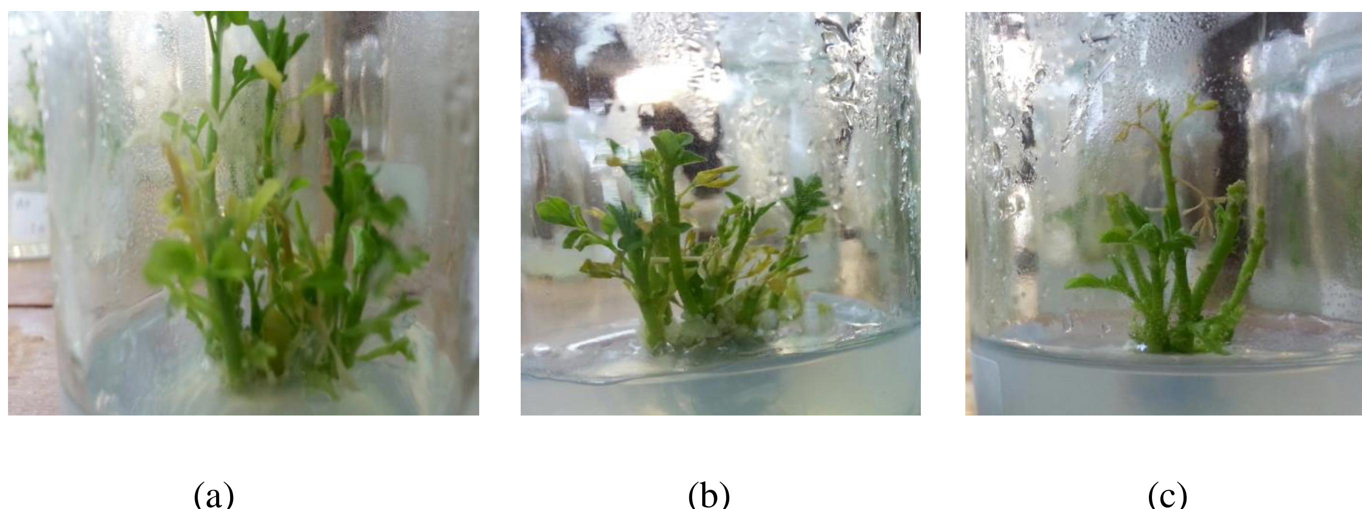

Figure 3. One week old multiple shoots were treated with UV-C irradiation from the UV lamp for (a) 5 min, (b) 10 min and (c) 15 min.

(2008). The UV-C illumination device consisted of an unfiltered germicidal UV lamp (El series UV-C lamp, UVP model UVS-28, Holland) located 29 cm above the radiation vessel. The UV-C intensities at the irradiation area were determined by using a UV-C light meter (Model: UV-C-254SD, Lutron Electronic, Germany). Peak radiation region was at approximately 254 nm. The schematic diagram is shown in Figure 2. The different UV-C illumination doses were obtained by altering the duration of the exposure at a fixed distance, that is 5, 10 and 15 min. Prior to use, the UV lamps were allowed to stabilize by turning them on for 15 min. A non-illuminated sample was considered as the control treatment. After illumination, samples were cultured to maintain the temperature at 25±3°C with 16 h photoperiod (40 μmol/m^2/s) with mercury fluorescent lamps for four weeks (Figure 3).

Fresh multiple shoot extraction

The fresh multiple shoots were minced into small pieces, weighed, and macerated in methanol for 72 h at room temperature (37±2°C) with occasional shaking. The extracts were centrifuged at 650 rpm for 6 min. The supernatants were stored at -20ºC until analysis.

Antioxidant activities determination

DPPH scavenging assay

The antioxidant activities were determined by DPPH scavenging assay using the procedure adapted from Vongsak et al. (2013). The anti-

oxidant activities of the extracts and trolox (standard solutions) were investigated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, USA). A total of 500 µL of the extract or of the standard was added to 500 µL of DPPH in methanol solution (152 µM). After incubation at room temperature for 20 min, the absorbance of each solution was determined at 517 nm using a UV-VIS spectrophotometer (Perkin Elmer, USA). The corresponding blank readings were also taken and the inhibition percentage was then calculated as follows:

$$\% \text{ Inhibition} = \frac{(A_1 - A_2)}{A_1} \times 100$$

Where, A_1 was the absorbance of the control reaction (containing all reagents except the test compound) A_2 was the absorbance of the test compound.

The concentration of sample required for 50% scavenging of the DPPH free radical value (IC_{50}) was determined from the curve of scavenging percentage plotted against the concentration of test compound or standard.

ABTS scavenging assay

The antioxidant activities were determined by ABTS scavenging assay using the procedure adapted method from Arnao et al. (2001). The stock solutions contained 7.4 mM $ABTS^{\bullet+}$(2,2'-azino-bis (3-ethylbenzothiazoline- 6 -sulfonic acid) diammonium salt radical solution) and 2.6 mM potassium persulfate solution. The working solution was then prepared by mixing 7.4 mM $ABTS^{\bullet+}$ solution and 2.6 mM potassium persulfate solution (1:1) and allowing them to react for 12 h at room temperature in the dark.

The solution was then diluted by mixing 1 mL $ABTS^{\bullet+}$ solution with 24 mL methanol to obtain an absorbance of 1.1±0.02 units at 734 nm using the UV-VIS spectrophotometer. Fresh $ABTS^{\bullet+}$ solution was prepared for each assay. Extracted samples (150 µL) were allowed to react with 2,850 mL of the $ABTS^{\bullet+}$ solution for 2 h in a dark condition.

Then the absorbance was taken at 734 nm using the UV-VIS spectrophotometer. The standard curve was linear between 25 and 600 mM trolox. Results were expressed in milligrams of trolox equivalents antioxidant capacity (TEAC)/100 g. fresh weight (FW). Additional dilution was needed if the ABTS value measured was over the linear range of the standard curve.

Ferric reducing power (FRP) method

The FRP method was adapted from Ferreira et al. (2007). The 250 µL extracted samples were mixed with 250 µL of 0.2 M sodium phosphate buffer and 250 µL of 1% (w/v) potassium ferric cyanide solution and then incubated at 50°C for 20 min. The mixtures were added with 1 ml of 10% (w/v) trichloro acetic acid and centrifuged at 650 rpm for 10 min.

The 250 µL supernatant was drawn and mixed with 250 µL of deionized water and 50 µL of 0.1% (w/v) ferric chloride solution. The absorbance of the mixtures was measured at 700 nm using the UV-VIS spectrophotometer. The content of Fe^{2+} was evaluated and expressed as mM $FeSO_4$ /100 g FW.

Total phenolics content determination

The total phenolic content was determined by the method adapted from Vongsak et al. (2013) using the folin-ciocalteu reagent. Each 100 µL of the 0.2 g/mL (w/v) samples was mixed with the 250 µL folin-ciocalteu reagent (diluted 1:10) with deionized water and 400 µL of 7.5% (w/v) sodium bicarbonate solution. The mixture was allowed to stand for 30 min at room temperature with intermittent shaking. The absorbance was measured at 765 nm using a UV-VIS spectrophotometer. The total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) / 100 g FW.

Total flavonoids content determination

The total flavonoid content was determined by the method adapted from Vongsak et al. (2013) using aluminum chloride. Each 400 µL of 0.04 g/mL (w/v) samples was mixed with 400 µL of 2% aluminum chloride solution. The mixture was allowed to stand for 10 min at room temperature with intermittent shaking. The absorbance of the mixture was measured at 415 nm against a blank sample without aluminum chloride using the UV-VIS spectrophotometer. The total flavonoids content was expressed as milligrams quercetin equivalent (QE)/100 g FW.

Quantitative analysis of major active compounds by HPLC

HPLC was performed on an Agilent 1260 series equipped with a quaternary pump 1260 Quat Pump VL, auto-sampler 1260 ALS, column thermostat 1260 TCC, and diode array detector 1260 DAD VL. The separation was carried out on a Hypersil BDS C-18 column (4.6 x 100 mm i.d., 3 µm) with a C-18 guard column. The mobile phase was 0.5% acetic acid (A) and methanol (B). The gradient elution was performed from 10% to 70% B in A for 20 min, and 100% B for 10 min. The column was equilibrated with 10% B in A for 10 min prior to each analysis. The flow rate was 1.0 mL/min at 25°C. The DAD detector was monitored at a wavelength of 334 nm for crypto-chlorogenic acid and 360 nm for isoquercetin and astagalin detection. The injection volumes for all samples including the standards were 20 µL.

Data collection and analysis

Each treatment was three replicates and the completely

Table 1. Antioxidant activities, total phenolic and flavonoid contents of shoot extraction of *M. oleifera* in the natural group, control group and experimental groups.

Treatment	DPPH assay (IC_{50}) (mg/mL)	ABTS assay mg TEAC/100 g FW)	FRP assay (mM $FeSO_4$ /100g FW)	Total phenolics (mg.GAE/ 100g FW)	Total flavonoids (mg.QE /100 g. FW)
Natural	45.69±0.62[d]	33.62±1.83[c]	18.10±0.80[c]	46.62±2.26[c]	37.83±0.98[c]
Control	44.29±0.56[d]	36.78±0.88[c]	20.70±0.38[c]	70.67±1.61[b]	41.52±0.85[c]
UV-C 5	40.89±0.56[c]	37.19±0.59[c]	22.52±0.74[bc]	73.48±1.64[b]	41.99±1.50[c]
UV-C 10	31.43±0.78[a]	58.98±2.09[a]	33.78±2.75[a]	112.56±2.94[a]	65.31±2.07[a]
UV-15	38.28±0.99[b]	50.22±0.63[b]	25.33±0.60[b]	81.34±1.14[b]	49.29±0.44[b]

[a,b,c,d] Dissimilar letters in the same column indicate a significant different at $p < 0.05$ using one-way ANOVA.

randomized design (CRD) was carried out. The data was analyzed using analysis of variance (ANOVA) followed by Duncan's multiple range test for the mean comparison.

RESULTS AND DISCUSSION

The antioxidant activities of *M. oleifera* in the natural group, the control group (UV-C for 0 min) and the experimental groups (UV-C for 5, 10 and 15 min) determined by the DPPH scavenging, ABTS scavenging and FRP assay showed that UV-C irradiation for 10 min showed the highest value with IC_{50} of 31.43 mg / mL, 58.98 mg TEAC/100 g FW (y =1.485x +7.104, R^2 =0.994, where y is percentage inhibition and x is concentration of trolox in µg/mL) and 33.78 mM $FeSO_4$ equivalents /100 g FW (y=0.000x - 0.016, R^2 =0.998, where y is the absorbance unit of Fe^{2+} and x is the concentration of Fe^{2+}), respectively (Table 1). The antioxidant activities of *M. oleifera* detected by these three assays were statistically significantly different among each treatment ($p < 0.05$).

Total phenolic and flavonoid contents determination showed that UV-C irradiation for 10 min had the highest value at 112.56 mg GAE /100 g FW (y = 0.036x + 0.143-, R^2 = 0.996, where y is the absorbance unit of gallic acid and x is the concentration of gallic acid in µg/mL) and 65.31 mg QE/100 g FW (y = 0.033x - 0.022 ; R^2=0.998, where y is absorbance unit of quercetin and x is concentration of quercetin in µg/mL)., respectively (Table 1). The total phenolic and flavonoid contents of *M. oleifera* detected were statistically significantly different among each treatment ($p<0.05$).

The higher antioxidant properties of the control group than that of the natural group might be explained by the plant growth regulator in the MS medium containing 0.5 mg/L BA enhancing the antioxidant activities as well as total phenolic and flavonoid contents. Our finding is consistent with the previous work of Suriyapan and Machachip (2009), Fedoreyer et al. (2000) and Mischenko et al. (1999) and Polsak (2003). As described, UV-C irradiation as the elicitor to cause oxidative stress (Benhamou, 1996) that could increase the biosynthesis of an important secondary metabolite such as antioxidative components, and stimulate the activities of the superoxide dismutase, catalase and peroxidase enzyme (Dornenberg and Knorr, 1995; Benhamou, 1996; Erkan et al., 2008). They could prevent and reduce cell and DNA damage from oxidative stress (Pongprasert et al., 2011) and UV-C irradiation at 3.6 KJ/m^2 could inhibit the declining of vitamin C and carotenoids of *Brassica alboglabra* var. *alboglabra* (Burana and Srilaong, 2009). UV-C irradiation was used to stimulate the production of antioxidants in the broccoli (Costa et al., 2006) and increase anthocyanin product in the callus culture of Ceylon spinach (*Basella rubra* Linn.) (Pumchaosuan and Wongroung, 2008).

Our findings show that UV-C irradiation for 10 min was the optimum duration to increase antioxidant activities which resulted in higher growth of *M. oleifera.* Applying UV-C irradiation for 5 min was too short to stimulate the antioxidant activities of *M. oleifera* while applying for 15 min was so long that it damaged cells and caused harm to *M. oleifera*.

The antioxidant bioactive components including crypto-chlorogenic acid, isoquercetin and astragalin was analyzed by HPLC using the modified method from Vongsak et al. (2012). The results showed that UV-C irradiation for 10 min had the highest value at 30.10 ng / mL (as determined by calibration curve: y=0.026x+1.264; R^2=0.999, where y is peak area and x is the concentration in ng/mL), 61.21 ng/mL (as determined by calibration curve: y =0.052x - 2.557; R^2=0.999, where y is peak area and x is the concentration in ng/mL) and 12.67 ng / mL (as determined by calibration curve: y=0.046x - 2.704; R^2=0.999, where y is peak area and x is the concentration in ng/mL), respectively (Table 2 and Figure 4). The crypto-chlorogenic acid, isoquercetin and astragalin determined were statistically significant different ($p<0.05$) among each treatment. UV-C irradiation for 10 min caused the total phenolic and flavonoid contents to be higher than those of the control group and the natural group as well as the antioxidative components crypto-chlorogenic acid, isoquercetin and

Table 2. Contents of crypto-chlorogenic acid (Cryp), isoquercetin (Iso) and astragalin (Astra) by HPLC (ng/mL).

Treatment	Cryp	Iso	Astra
Natural	4.49±1.30[c]	15.53±1.36[c]	4.54±0.72[c]
Control	4.53±1.11[c]	31.99±3.72[b]	5.38±0.94[c]
UV-C 5	14.40±4.09[b]	36.58±2.60[b]	8.90±3.07[b]
UV-C 10	30.10±3.88[a]	61.21±4.70[a]	12.67±5.40[a]
UV-C 15	15.61±2.72[b]	37.82±4.87[b]	9.13±2.02[b]

[a,b,c,d]Dissimilar letters in the same column indicate a significant difference at $p < 0.05$ using one-way ANOVA.

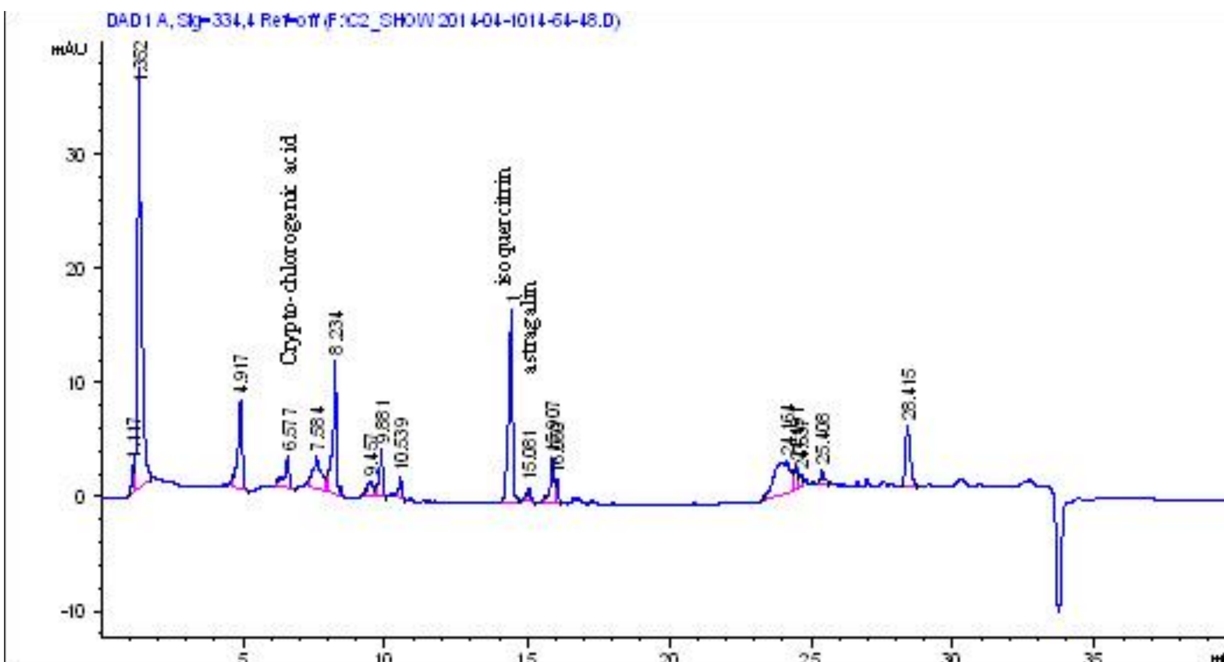

Figure 4. HPLC chromatogram showing the *Moringa oleifera* shoot culture profile.

astragalin. It can be concluded that UV-C irradiation could stimulate the activities of the enzyme in the biosynthesis of the antioxidative compounds resulting in increasing antioxidant capacities of *M. oleifera*.

Conclusion

UV-C irradiation for 10 min was the optimal duration for stimulating antioxidant capacities and antioxidant compounds in shoot cultures of *M. oleifera,* resulting in higher antioxidant activities, total phenolic and flavonoid contents as well as the amount of antioxidative components crypto-chlorogenic acid, isoquercetin and astargalin than those of the control group and the natural group. UV-C irradiation may also stimulate the activities of the superoxide dismutase, catalase and peroxidase enzyme that are involved in the biosynthesis of antioxidative compounds. Our study could provide useful knowledge that can be utilized for improving the quality of *M. oleifera* raw materials in herbal supplementary food and medical uses.

Conflict of Interests

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

ACKNOWLEDGEMENTS

This research was financially supported by National Institute of research, Thailand and Uttaradit Rajabhat University, Thailand.

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