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Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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Review

Biogenesis of nanoparticles: A review

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Advancement in nanotechnology mainly depends upon advancement in nanomaterial. There are many chemical routes known to use toxic chemicals for synthesis of nanoparticles but the need of the hour is to use environmental benign, greener and safer routes. Researchers are looking to use various living organisms as 'nanoparticle factories'. Various biological entities like bacteria, fungi, diatoms, higher plants, actinomycetes and viruses have been used for this purpose. Due to their normal biosynthetic pathways, they can reduce salt into corresponding nanoparticles. This review includes some of the biological sources which have been used by researchers for the synthesis of nanoparticles and their applications.

Key words: Biogenesis, nanofactories, nanoparticles, antimicrobial activity, semiconductor nanoparticles.

INTRODUCTIONS

The field of nanotechnology has generated great enthusiasm in recent years because of its expected impact on science, industry, economy and our everyday life. Nanomaterial synthesis, characterization and its manipulation is one of the major aspects of nanotechnology (Bansal and Suresh, 2012). Nanoparticles (NPs) have unique properties as compared to their bulk equivalents, thus find application in a number of fields including optics (Kawar, 2011), electronics, sensor technology, clinical biology (Gupta, 2011), catalysis etc. With the advancement in techniques now, we are moving towards 'green synthesis' of nanoparticle production (Bansal et al., 2012).

Besides the traditional, physical and chemical methods of nanoparticles synthesis, biogenesis of nanoparticles is also gaining atten-tion of researchers due to some clear advantages of using biological entities for NPs production. Physical (Ayyub et al., 2001; Kalishwaralal et al., 2010) and chemical methods (Murray et al., 2002) of production like gel-sol synthesis, chemical reduction method, aerosol technology, lithography, laser ablation method are expensive and involve the use of hazardous chemicals and reagents that pollute environment when we talk about their bulk production. Nature has devised various processes for the synthesis of nano- and micro-length scaled inorganic materials which have contributed to the development of relatively new and unexplored area of research based on the biosynthesis of nanomaterials (Mohanpuria et al., 2007). Microbes may solublize the metals or can reduce them. Microbial biomass can retain relatively high quantities of metal by biosorption (passive mode) or by bioaccumulation (actively by viable cells)

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(Volesky, 1995). Biogenesis of nanoparticles is one of such environmental friendly approach as it makes use of organisms and their natural biochemical mechanism (Simkiss, 1989; Mann, 1996) for production of nanoparticles thus, provides an economical and safer route. Moreover, the biologically fabricated nanostructures offer substantially different in properties like good adhesion, tribologically good properties, less toxicity and biocompatibility making them more valuable for biological applications. It is interesting to note that a range of organisms including bacteria, fungi and higher plants show remarkable synthesis of nanoparticles thus acting as 'nanofactories'.

BACTERIA

It has been shown that many bacteria can actively uptake and reduce metal ions. Microbes can either show oxidization, reduction or biosorption of metals. The production of nanoparticle can be extracellular or intracellular depending upon bacterial species. There is a limit of nanoparticle accumulation up to which bacteria can survive and after that, nanoparticle accumulation can be toxic for the microbes (Deepak et al., 2011). First evidence of silver nanoparticle production came from bacteria *Pseudomonis stuzeri* AG 259 which was isolated from silver mine. When placed in silver nitrate solution, it produces NPs in its periplasmic space which are of pyramidal and hexagonal shape and size up to 200 nm (Klaus et al., 1999; Joerger et al., 2000). Synthesis of extracellular silver nanoparticles at room temperature was studied in *Pseudomonas aeruginosa* strain BS-161R and its antimicrobial efficiency was studied against different Gram positive, Gram negative and *Candida* species (Kumar and Mamidyala, 2011).

Nithya et al. (2011) studied silver hydrosol formation using three bacterial strains; that is, *Streptococcus* sp, *Proteus* sp and *Pseudomonas* sp. Within 30 min, all the three strains started showing change in color. After 24 h, color of bacterial filtrate changed from pale yellow to brown indicating formation of silver nanoparticles. Extracellular production of silver metal nanoparticles by probiotic microbes (*Brevibacterium linens* NCIM 2149) using 1 mM $AgNO_3$ solution has been reported by Nithya and Raghunathan (2012) and the synthesized NPs showed very good antimicrobial activity against multidrug resistant (MDR) Gram negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*. Applications of silver nanoparticles and its toxicity have been studied by different researchers (Prabhu and Poulose, 2012). Silver (Ag) NPs were synthesized by using different root nodule microbes including *Rhizobium* and *Agrobacterium* by Rajkumar and Tamizharasi (2012) and its antimicrobial activity against different pathogens *E. coli*, *Vibrio cholerrae, Proteus vulgaris* was checked. Silver (Ag) NPs showed no inhibition on *P. vulgaris* but showed good inhibition on other two bacteria at concentration of 50 µl. Minaeian et al. (2008) investigated Ag NPs synthesis by using a range of bacterial species *Klebsiella pneumoniae, E. coli, Enterobacter cloacae, Staphylococcus aureus, Bacillus subtilis, Lactobacillus acidophilus* and *Candida albicans* using silver nitrate at concentration of 10^{-3} M. Among these *K. pneumoniae, E. coli* and *E. cloacae* were found effective for extracellular production of Ag NPs. Potential of nine different *Lactobacillus* strains, extracted from whey, for Ag nanoparticle synthesis was studied. *Lactobacillus* sp VRS-2, showed maximum potential with nanoparticle of size 2 to 20 nm (Rangnathan et al., 2012). Biomass of *Bacillus cereus* isolated from leaves of surface sterilized medicinal plant *Garcinia xanthochymus* synthesized silver nanoparticles by reducing $AgNO_3$ at room temperature in three to five days (Sunkar and Nachiyar, 2012). *Vibrio alginolyticus* reported to produce intracellular and extracellular spherical Ag NPs depending upon procedure followed by Rajeshkumar (2013). Intracellular reaction started in 4 h and extracellular reaction took 12 h. Extracellular synthesis showed better dispersibity and control over size of synthesized NP.

Gold nanoparticles show very high chemical reactivity as compared to bulk gold which is comparatively inert. Recent study on biosynthesis of gold nanoparticles by *Geobacillus* sp. strain ID17, a thermophilic bacterium isolated from deception Island, Antarctica done by exposing bacterial cells to Au^{3+} ions (Correa-Lianten, 2013). Other bacteria for gold nanoparticle synthesis include *E. coli* DH5a and their applications on direct electrochemistry of hemoglobin are reported (Du et al., 2007). *Rhodococcus*, alkalo-tolerant actinomycetes synthesized intracellular gold nanoparticles of uniform size 5 to 15 nm and keep on dividing even after accumulation of gold nanoparticles on its cell wall and cytoplasmic space. Thus, confirming non toxicity of gold nanoparticles against *Rhodococcus* (Ahmad et al., 2003). Effect of pH on shape of gold NPs has been reported. At pH 4, nanoplates were obtained and at pH 7 spherical nanoparticles were accumulated (He et al., 2008).

A well studied example of NPs production is magnetosome. These are either Fe_3O_4 (magnetite) or Fe_3S_4 (greigite) nanoparticles in the magneto-tactic bacteria such as *Magnetospirillium magneticum* that can synthesize magnetic nanoparticles (Samuel, 2005; Bazylinski and Frankel, 2004) which have an enormous number of applications in bio and nanotechnology (Lang et al., 2006). Their superior nature allows magnetosomes to be used as magnetic nanoparticles. Semiconductor nanoparticles have exceptional optical and electronic properties and hence, can be of great importance for opto-electronic devices. Under anaerobic conditions, using N-8 and M-8 culture media, Mandal et al. (1998) observed ZnS nanoparticles of 2 to 5 nm produced by the

sulphate reducing bacteria *C. vulgaris*. Sulphate reducing bacterium *Desulfovibrio desulfuridcans* NCIMB 8307 and *Shewanella oneiclensis* has been shown to produce palladium nanoparticles, which find application in catalysis (Yong et al., 2002; Windt et al., 2005). A highly cadmium resistant strain *Klebsiella planticola* Cd-1 isolated from reducing salt marsh sediments, precipitated considerable amount of CdS nanoparticles (Sharma et al., 2000). *K. pneumonia*e synthesized stable selenium nanoparticles in elemental form from selenium chloride and were found stable against wet heat sterilization. Thus, wet heat sterilization was done to separate selenium nanoparticles from bacteria (Fesharaki et al., 2010). Precipitation of palladium nanoparticles on bacteria *Citrobacter braakii* to obtain palladium nanoparticles and its application as catalyst for diatrizoate removal with biogenic hydrogen was successfully studied by Hennebel et al. (2011). *Lactobacillus* mediated synthesis of silver oxide nanoparticle of size up to 2 nm has been studied by Dhoondia et al. (2012). CdS NPs synthesized by sulfur reducing bacteria *Serratia nematodiphila*, isolated from chemical effluent of a company has been reported by Malarkodi et al. (2013).

Newman et al. (1997) reported that *Desulfosporo auripigmenti* can precipitate spherical, arsenic trisulfide nanoparticles (As_2S_3), both intra and extracellular under sulfate-reducing conditions. The microbial production of extracellular network filamentous arsenic sulphide (As-S) nanotubes by *Shewanella* sp has been reported by Jiang et al. (2009). Aerobically, growing *Duganella sp.* and *Agrobacterium sp.* from selenium contaminated agriculture soils of Punjab (India) has been isolated and found to be effective in converting bio-soluble toxic selenium anion to non toxic elemental selenium NPs (Bajaj et al., 2012), thus, showing its potential in controlling biogeochemical cycle of Se in natural environment. Ion chromatographic analysis released only reduction of Se (IV) to Se (0) and no oxidation. *Duganella* found to be more efficient for producing NPs.

FUNGI

Fungi have some distinct advantages when used as nanofactories for NP production (Volsky and Holan, 1995)

1. Easy to scale up, large biomass can be obtained as fungi grow easily on large scale by solid state fermentation method.
2. Large scale production of extra cellular enzyme per unit biomass.
3. Ease of biomass handling.
4. Most fungi have a very high wall-binding capacity as well as intracellular metal uptake capacities.
5. Good metal accumulation capability.

The biosynthesis of intracellular quantum crystallites in yeast has been reported in *Candida glabarata* and *Schizosaccharomyces pombe* when cultured in the presence of cadmium salt (Dameron et al., 1989). Extracellular synthesis of silver nanoparticles was observed in silver tolerant yeast strains MKY3 when exposed with 1 mM soluble silver in the log phase of growth (Kowshik et al., 2003). Potential of *Aspergillus fumigatus* for extracellular synthesis of mono-dispersed Ag NPs has been explored (Bhainsa and D'Souza, 2006). Crystallized and spherical-shaped Au and Au-Ag alloy nanoparticles have been synthesized and stabilized using a fungus, *Fusarium semitectum* in an aqueous system. Aqueous solutions of chloroaurate ions for Au and chloroaurate and Ag^+ ions (1:1 ratio) for Au-Ag alloy were treated with an extracellular filtrate of *F. semitectum* biomass for the formation of Au nanoparticles (Au NP) and Au-Ag alloy nanoparticles (Au-Ag NP) (Balaji et al., 2008). Extracellular silver nanoparticles of size 5 to 50 nm were obtained by use of *Pleurotus sajorcaju* and their antimicrobial activity have been recently reported by Nithya et al. (2009).

Ray et al. (2011) showed for the first time the green biosynthesis of silver nanoparticles form the mycorrhizal fungus *Tricholoma crassum* (Berk.) Sacc. The spherical silver nanoparticles were of the size range 5 to 50 nm. These silver nanoparticles showed potent antimicrobial activity against multidrug resistant pathogenic bacteria *E. coli* (DH5α), plant pathogenic bacteria *Agrobacterium tumifaciens* (LBA4404) and plant pathogenic fungus *Magnaporthe oryzae*. Room temperature synthesis of Ag NPs from *Penicillium* extracted from field soil has been studied and mechanism of nanoparticle synthesis has also been established. Pure filamentous *Penicillium* is allowed to react with 1 mM $AgNO_3$ to obtain optimum reduction process (Hemath et al., 2010). Freeze dried *Phoma sp.* 3.2883 mycelia when treated with $AgNO_3$ solution in shaking conditions synthesized good amount of Ag NPs (Chen et al., 2003). The presence of hydrogenase in the *Fusarium oxysporum* has been demonstrated. This extracellular enzyme shows excellent redox properties and it can act as an electron shuttle in metal reduction. Mukherjee et al. (2001) demonstrated that the fungus *Verticillium* sp. when subjected with an aqueous solution of chloroaurate ($AuCl_4$) resulted in the reduction and intracellular accumulation of gold nanoparticles with average size of around 17 nm. The capability of *F. oxysporum* to synthesize gold nanoparticle by treatment of the fungal biomass with aqueous solution containing $AuCl_4$ ions was further demonstrated by Mukherjee et al. (2002). The fungus *Verticillium sp*. produces nanoparticles, when exposed to gold and silver ions; metal ions were reduced fairly by biomass and formed respective metallic nanoparticles.

This fungus produces intracellular nanoparticles (Senapati et al., 2004). Live biomass of fungus *Penicillium brevicompactum* successfully used for synthesis of gold nanoparticles and cytotoxic effects of synthesized nanoparticles were studied on mouse mayo blast cancer C2 C12 cells (Mishra et al., 2011). Synthesis of Au NPs on the surface of *Rhizopus oryzae* by *in situ* reduction of $HAuCl_4$ and its application in water hygiene management has been studied (Das, 2009).

Aspergillus tubingensis and *Bionectria ochroleuca* mediated synthesis of extracellular silver nanoparticles, which are spherical in shape with dimensions of 35 $\pm$ 10 nm was studied by (Rodreguis et al., 2013). *A. tubingensis* synthesized NPs showed excellent antimicrobial activity and very high surface positive potential as compared to other fungi studied so far. Zirconia nanoparticles have been produced by exposing the fungus *F. oxysporum* with aqueous ZrF_6^{2-} anions and extra-cellular protein-mediated hydrolysis of the anionic complexes results in the novel room temperature synthesis of zirconium nanocrystals (Bansal et al., 2004). Kowshik et al. (2002) reported the intracellular synthesis of cadmium sulfide nanoparticles by *S. pombe* strain. In another work, using *Fusarium* sp. biomass as a sustainable synthesis procedure, CdS NPs has been synthesized after its exposition with a $CdSO_4$ solution (Ahmad, 2002; Reyes et al., 2009).

ALGAE

The extracellular biosynthesis of silver nanoparticles using marine cyanobacterium *Oscillatory willei NTDMO1* which reduce silver ions and stabilizes the silver nanoparticles by a secreted protein was recently reported (Ali et al., 2011). An efficient approach for synthesis of stable gold nanoparticles by the reduction of aqueous $AuCl_4$ using *Sargassum wightii* was reported (Singaravelu et al., 2007). It was the first report for synthesis of stable metallic nanoparticles by the extract of marine algae. Biological synthesis of gold nanoparticles within 10 min by brown alga, *Stoechospermum marginatum* biomasses through a green route was reported by (Arockia et al., 2012). *Spirulina platensis* biomass directed synthesis of Ag NPs of approximately 12 nm has been successfully achieved and size was confirmed by XRD (Mahdieh et al., 2012). *Nannochloropsis oculata, Dunaliella salina* and *Chlorella vulgaris* as three algal species in addition to three *Lactobacilli* including *Lactobacillus acidophilus, Lactobacillus casei* and *Lactobacillus reuteri* were monitored for their potential of silver nanoparticle synthesis. The biosynthesis of silver nanoparticles in all three *Lactobacilli* and two algal species *N. oculata* and *C. vulgaris* was confirmed (Mohseniazar et al., 2012). Recently, copper oxide NPs had been synthesized by brown algae *Bifurcaria bifurcate* and were found to be effective against two different strains of bacteria *Enterobacter aerogenes* and *Staphylococcus aureus* (Abboud et al., 2013).

VIRUSES

Viruses do not synthesize nanoparticles as such, but there are several reports on use of viruses for template dependent synthesis of nanoparticles. Viroid capsules (Douglas et al., 1998) are used in template mediated production of inorganic nanomaterials and microstructured materials. Tobacco mosaic virus (TMV) has been successfully used as template for the synthesis of iron oxides by oxidative hydrolysis, co-crystallization and mineralization of CdS and lead sulphide (PbS) crystalline nanowire. A hybrid nanowire (ZnS-CdS) is obtained with a dual peptide virus engineered to express A7 and J140 within the same viral capsid (Shenton et al., 1999; Mao et al., 2003).

PLANT AND PLANT EXTRACTS

The plant mediated synthesis of nanoparticles is more advantageous than the other biological process as there is no troublesome of preserving and maintaining the cell culture. Plant mediated nanoparticle synthesis is an easy, one step synthesis method with no chances of mutation as in microorganisms. Extraction and separation can be easily scaled up for the large-scale synthesis of NPs (Veerasamy et al., 2011). By using a natural plant reducing constituent geraniol, extracted from different plants, silver NPs up to 10 nm size range were synthesized with average particle size of 6 nm and cytotoxicity studies of synthesized NPs were done by Safaepour et al. (2009). It was the first time when only the active component from plant was used for biosynthesis of NPs.

Synthesis of anisotropic gold and spherical-quasi-spherical silver nanoparticles by reducing aqueous chloroauric acid and silver nitrate solution at room temperature was studied and it was found that size and shape of the nanoparticles can be controlled by varying the concentration of phyllanthin extract (Kasthuri et al., 2009). *Cinnamon zeylanicum* bark extract, upon evaluation for synthesis resulted in cubic and hexagonal silver nanocrystals with size ranging between 31 to 40 nm (Sathishkumar et al., 2009). A report synthesis of AuNPs using *Gnidia glauca* flower extract (GGFE) and its evaluation of chemocatalytic potential under different environmental conditions were used to study optimal conditions and it was found that 0.7 mM $HAuCl_4$ at 50°C is optimum for maximum production (Ghosh et al., 2012). By using extract of *Rosmarinus officinalis,* silver NPs of size 60 nm and its antimicrobial activity against pathogens

Table 1. List of some higher plants reported for production of NPs.

Plant	Synthesized nanoparticle	Reference
Azadirachta indica leaf extract	Gold, silver and gold core silver shell	Shankar et al., 2004
Aloe vera extract	Gold nanotriangles and silver NPs	Chandran et al., 2005
Coriander leaf extract	Gold NPs	Narayanan et al., 2008
Honey	Gold NPs	Philip et al., 2009a,b
Syzygium aromaticum	Gold NPs	Deshpande et al., 2010
Moringa oleifera	Silver	Prasad et al.,2011
	Silver	Mubayi et al., 2012
Ocimum basilicum	Gold NPs	Singhal et al., 2012
Momordica charantia	Gold NPs	Pandey et al., 2012
Ficus benghalensis	Silver NPs	Saxena et al.,2012
Justicia gendarussa burm F leaf extract	Gold NPs	Fazaludeen et al., 2012

including *S. aureus* and *S. pneumonia* was reported by Sulaimana et al. (2013).

Iron and silver nanoparticles were synthesized at room temperature using a rapid, single step biosynthetic method employing aqueous *Sorghum* extracts as both the reducing and capping agent. Highly crystalline silver nanoparticles with an average diameter of 10 nm were obtained (Njagi et al., 2011). Geranium leaves (*Pelargonium graveolens*) and its endophytic fungus *Colletotrichum* sp used in the extra-cellular synthesis of gold nanoparticles. Leaves and fungus growing in the leaves were separately exposed to aqueous chloroaurate ions and in both cases, rapid reduction of the metal ions was observed resulting in the formation of stable gold nanoparticles of variable size (Shankar et al., 2003). Torresdey et al. (2002) reported the formation of gold nanoparticles in *Alfalfa* (*Medicago sativa*) plants. *Alfalfa* roots have ability of absorbing reduced silver ions Ag (0) from production medium and transferring it to shoot of the plant in the same state of oxidation. Inside the plant tissue, reactions occur and the accumulation of Ag NPs takes place. Another quick method of Ag NPs biogenesis was reported where *R. officinalis* extract was heated with 1 mM AgNO3 at 7°C for 3 min. The obtained NPs were tested for its antimicrobial potential against six different pathogens (Ghassan et al., 2013).

Copper NPs were synthesized using the latex of *Calotropis procera* by using copper acetate. Cysteine proteases in the latex are responsible for the reduction of copper ions. Cytotoxicity studies were performed on latex stabilized copper nanoparticle on HeLa, A549 and BHK21 cell lines by MTT dye conversion assay and Cu NPs, thus synthesized were excellent in terms of biocompatibility (Harne et al., 2012). Biosynthesis of copper (Cu), zero valent iron (ZVI), silver (Ag) nanoparticles using leaf extract of *Dodonaea viscosa* has been investigated by Daniel et al. (2013). The synthesized spherical nanoparticles showed the average size of 29, 27 and 16 nm for Cu, ZVI, and Ag nanoparticles, respectively. Biosynthesized Cu, ZVI, and Ag nanoparticles were tested against various human pathogens viz. Gram-negative *E. coli*, *K. pneumonia*, *Pseudomonas fluorescens* and Gram-positive *S. aureus* and *B. subtilis* and showed good antimicrobial activity. Some other higher plants which produced NPs are shown in the Table 1.

Diatoms are single celled photosynthesizing algae. It has been shown that the freshwater diatom, S*tauroneis* sp. can be used to fabricate silicon - germanium oxide nanoparticles by a two step process (Ali et al., 2011). Mono-dispersed platinum and palladium nanoparticles of size up to 6 nm have been synthesized at room temperature by using root extract of *Asparagus racemosus* (Linn.) (Raut et al., 2013). Natural hydrocolloids isolated from trees are new class of potentially economical and environmental friendly biomaterial that exhibits a high specificity for the production of nanomaterials. Gums extracted from plants may act as both reducing and capping agents in nanoparticle synthesis. Vellora et al. (2013) synthesized CuO nanoparticles using gum karaya, a natural nontoxic hydrocolloid that explored its antimicrobial properties.

CONCLUSION

Biologically synthesized nanoparticles are gaining attention day by day in various fields including electronics, optics, medicines and chemical industries. Due to simplicity, cost effectiveness and eco friendly nature of this method, biogenesis of NPs is of much interest for future prospective. Range of biological entities including bacteria, fungus, viruses, algae, viruses including higher plants has been used so far. Production can be classified as intracellular and extracellular on the basis of site of NP accumulation in biological entity. By controlling different physical parameters like temperature, pH, media, reaction time etc. shape, size and quantity of NPs can be controlled. Different metallic, non metallic and semiconductor NPs have been produced and their

antimicrobial activity has been studied over different pathogens. As compared to physical and chemical methods, biogenesis of NP is time consuming process but it is much cheaper and environmental friendly. Moreover when we talk about biological applications biogenetically synthesized NPs are supposed to be free of toxic chemicals and hence, supposed to be more compatible with biological entities and safer to use. Still much refinement is needed for using this method for large scale synthesis of NPs in terms of efficiency and control over shape and size.

Conflict of interests

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

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Review

Genetic diversity and conservation of *Picea chihuahuana* Martínez: A review

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The conservation of genetic diversity in tree populations is an essential component of sustainable forest management. *Picea chihuahuana* Martínez is an endemic conifer species in Mexico and is considered to be endangered. *P. chihuahuana* covers a total area of no more than 300 ha at the Sierra Madre Occidental, a mountain range that harbor a high diversity of tree species. There are 40 populations of the species that have been identified in the region, and it cannot be found elsewhere. These populations form clusters within gallery forests and are usually associated with eight other tree genera. The *P. chihuahuana* community is mostly well preserved. Owing to its remarkable characteristics and high conservation value, *P. chihuahuana* has been the subject of several studies aimed at learning more about the genetic structure, ecology and potential effects of climate change. However, the overall applicability of such studies is to confirm a dataset to develop management tools to help decision makers and to implement preservation and conservation strategies using genetic diversity. In this review, we summarize the studies carried out to date, emphasizing those concerning the most important aspects of the genetic diversity of the species. Although, genetic diversity in Chihuahua spruce is mostly moderate compared with other *Picea*, this species is unlikely to survive without help due to its small and isolated populations. Efforts should focus on the protection of populations displaying the highest degree of genetic variation because these populations have the greatest potential for adaptive evolution. Finally, continuous monitoring of size and genetic diversity of the current populations *in situ* is essential.

Key words: Pinabete, spruce, endangered species, heterozygosity, genetic variability, Sierra Madre Occidental.

INTRODUCTION

In a broad sense, genetic variability or genetic diversity is a basic component of biodiversity and it is defined as inheritable variations occurring in every organism, among individuals within a population and among populations within a species. Hence, knowledge and understanding of genetic diversity is of vital importance for evolutionary

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genetics, as well as for public health, productivity and sustainability of agricultural, livestock, fishing and forestry industries, domestication and biomedicine (Piñero et al., 2008). Historical biological processes, such as fragmentation, expansion of the geographical distribution and events such as genetic bottlenecks and other responses to climatic and geological changes, have altered genetic diversity and molded patterns of genetic differentiation among and within species and populations (Ledig et al., 1997; Schaal et al., 1998). These events can be detected in the genetic structure of many tree species due to their extended generation times (Newton et al., 1999). Conservation of genetic diversity in tree populations is an essential component of sustainable forest management. The abilities of trees and other forest species to evolve, resist and adapt to climate change largely depend on the genetic diversity within species (St. Clair and Howe, 2011). The Intergovernmental Panel on Climate Change (IPCC) estimates that global average temperature will increase about 1.8 to 4.0°C during the 21st century. An average increase in temperature of 3.7°C and a decrease in precipitation of 18.2% by the end of the century are predicted for Mexico (Sáenz-Romero et al., 2010). Furthermore, the extinction risk will increase for 30% of the world's species (Intergovernmental Panel on Climate Change, 2007).

The study of genetic diversity can be applied to the following: a) evaluation of the responsiveness of populations and species facing environmental changes caused by anthropogenic activities; b) assessment of risks related to loss of species, populations and genetic resources; c) knowledge of the genetic richness and its geographical distribution; d) planning of exploitation and conservation strategies for populations, species and genetic resources; e) understanding the mechanisms, rate and causes of the loss of genetic diversity; f) assessment of risks produced in populations, native species and genetic resources of plants, animals and humans by introducing diseases, pests, invasive species and genetically improved varieties (Piñero et al., 2008). Until now, effects on genetic diversity originated by fragmentation, isolation and reduction of population size have been known theoretically. However, empirical data from natural populations of forest trees are relatively scarce. Wright's theory suggests that the smallest populations without gene flow will lose genetic variability faster than larger populations because small populations are more susceptible to such loss due to genetic drift, endogamy depression and strong unidirectional selection, causing genetic erosion (Wright, 1969; Karron et al., 1988; Maxted and Guarino, 2006; Wehenkel and Sáenz-Romero, 2012). Locations displaying high genetic diversity probably represent the populations that are most adaptable to changes; therefore, these locations are the most suitable for focusing conservation efforts (Ledig et al., 2005). However, some simulation studies suggest that variability may not be lost as quickly as Wright's (1969) model indicates and that selection of heterozygotes could delay the loss of alleles (Lesica and Allendorf, 1992). The study and interpretation of patterns of intraspecific genetic variation should be a priority for academic institutions, governmental and non-governmental organizations related to environmental conservation and sustainable natural resources management, in order to learn about dynamics, adaptation and evolution of the species (Ellstrand and Elam, 1993) and to contribute to the design of protection and conservation strategies for the species.

Studies of genetic diversity in Mexican plant species are scarce in relation to the number of studies on other aspects (e.g., ecological aspects) of the reported species in the country (CONAFOR, 2011). One exception to this is *Picea chihuahuana* Martínez of great value, because this spruce and two other congeners, *Picea mexicana* Martínez and *Picea martinezii* Patterson, are relicts of the last glaciation, and the only representatives of the genus *Picea* located in southern latitudes in North America (Ledig et al., 1997; Ledig et al., 2000a). This species has been the subject of several investigations aimed at learning more about the ecology, genetics, potential and association with other species. However, the main aim of such studies has been to enable the design of preservation and conservation strategies (Sánchez and Narváez, 1990), as discussed further below.

GENUS *PICEA*

Picea is a mainly boreal genus and, depending on the taxonomy used, includes 31 to 50 species (Wright, 1955; Bobrov, 1970; Farjon, 2010). The genus *Picea* occurs in Europe, Asia, the Caucasus, Siberia, China, Japan, the Himalayas, North America and Mexico. The genus has evolved from primordial ancestors in the continental part of Northeast Asia (Youngblood and Safford, 2002). The Korean fir *(Picea koraiensis* Nakai) is probably the most primitive species of this genus. Most North American species probably arose due to the eastward migration and mutation of some fir species (Wright, 1955). Fossilized pollen is a source of reference data for interpreting changes experienced by boreal forests over time. This information has enabled reconstruction of the variation of geographical boundaries for boreal species in Western Europe and North America (Hansen and Engstrom, 1985). The most characteristic feature is the northward shift of some species at the end of the last glaciation. Boreal forest communities reached their present configuration during the last 5,000 years. Boreal forest boundaries were altered at the end of the last glacial period at very different speeds and in different ways for each species. This variability indicates that each species reacts differently to environmental changes and the introduction of a similar tree species contributes to interspecific variability in response to future environmental changes

(Delcourt and Delcourt, 1987; MacDonald, 1992). Since the end of the last glaciation, there have been significant changes in the distribution of genetic diversity of some tree species. Because of this, future climate change due to global warming may cause a significant loss of genetic diversity in boreal plant species (Prentice et al., 1991). Other factors, such as deforestation and pollution, also endanger forest genetic diversity. These threats have affected the flora and fauna of the forests, as well as the habitats of various organisms. However, immediate loss of species is not the only danger, considering that many populations of the surviving species will disappear; because of this, much of the genetic diversity needed for the long-term survival and evolution of the species will be lost (Ledig, 1988).

A decrease during the Holocene warming (about 7,000 to 8,000 yr B.P. as suggest palynological evidence) in the number of *Picea* individuals in Mexico and their historical northward shift coincides with the interglacial global warming period (Ledig et al., 1997; Ledig et al., 2010). Recent studies indicate that Mexican endemic spruces are susceptible to climate change, because its suitable climate habitat will disappear too fast, and unless human assistance, they will not be able to reach (by natural means) the sites where it will occur at the remaining suitable climatic habitat, that in general will occur at distant places than the ones at present (Ledig et al., 2010).

The presence of genus *Picea* in Mexico's subtropical and tropical latitudes (although at very specific sites, generally on high altitudes, northern aspect, humid sites near of creeks; Ledig et al., 2000a) is highly unusual, because these sites are much more southerly than the usual distribution of spruces in North America. The native spruces from Canada and USA do not include the rare Mexican *P. chihuahuana* Martínez, which is located in Northwest Mexico (Patterson, 1988).

Picea chihuahuana Martínez

This species is commonly known as Pinabete or Pinabete espinoso. According to a Mexican Official Standard (Norma Oficial Mexicana, NOM-ECOL-059-2010), this is an Endangered species and an endemic relict (Gordon, 1968; Narváez, 1984; García-Arévalo, 2008). However, before the official status of endangered was determined, Sánchez and Narváez (1983, 1990) already considered the very rare and fragmented species as Endangered, using as criteria the restricted distribution of the species, as well as its special ecological conditions, which are mainly related to specific soil and vegetation characteristics. Gordon (1968) and Mata (2000) reported that the main causes of the status of this endangered species was geographically isolated populations, irregular reproduction due to heterogeneous age distributions in populations, scarce natural generation, and clandestine cutting down of complete juvenile trees and tree crown tops for Christmas trees.

P. chihuahuana is also included in the list of Endangered taxa collected by the National Institute of Livestock, Forest and Agricultural Research (Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias, INIFAP) (Vera, 1990), and it is classified as Endangered according to the guidelines of the International Union for the Conservation of Nature and Natural Resources, IUCN Red List because "subpopulations are severely fragmented and there is an ongoing decline due to fires, logging and grazing. ... The number of mature individuals is uncertain but could be less than 2,500".

Several factors lead to *P. chihuahuana* impoverishment, like reduction of its reproductive capacity due to high levels of self-fertilization and mating between closely related individuals. Harvesting, grazing and forest fires also contribute to reduction of population sizes (Ledig et al., 1997). However, currently wood logging is a marginal factor (Wehenkel and Sáenz-Romero, unpublished).

This species is usually associated with pines (*Pinus spp.*), oaks (*Quercus spp.*), and occasionally with species of the genera *Abies*, *Pseudotsuga, Cupressus, Populus Juniperus,* and *Prunus* (Gordon, 1968; Narváez et al., 1983; Quiñones-Pérez et al., 2014); it is also sometimes associated with other species of the same genus, such as *Picea engelmannii* and *Picea pungens* (Gordon, 1968). Individual trees reach heights of up to 50 m in height and diameters up to 120 cm (Ledig et al., 2000a). Lower branches are almost horizontal, starting at 2 to 5 m in height, while higher branches are extended and somewhat raised, forming a conical crown (Gordon, 1968).

Location of *P. chihuahuana* Martínez

The existence of the species in Mexico was unknown before 1942. *P. chihuahuana* was first discovered in the State of Chihuahua in Northwestern Mexico (Martínez, 1953). The species was subsequently found in other locations in Chihuahua and Durango. The recent discovery of the species is indicative of their rarity and inaccessibility (Gordon, 1968). In Chihuahua, *P. chihuahuana* is located in the municipalities of Bocoyna, Temosachi, Guerrero and Balleza; in Durango, it is found in the municipalities of Pueblo Nuevo, El Mezquital, San Dimas, Canelas and Guanaceví (Ledig et al., 2000a) (Figure 1).

P. chihuahuana covers a total area of no more than 300 ha and it is located at least 40 sites along the Sierra Madre Occidental in Durango and Chihuahua states (Table 1) (Sánchez, 1984; Farjon et al., 1993, Ledig et al., 2000a; Wehenkel and Sáenz-Romero, 2012), and a minimum total of 42,600 individuals has been estimated (Ledig et al., 2000a); these sites are located at medium altitudes, ranging from 2,150 to 2,990 m a.s.l., with average temperatures from 9 to 12°C. The species probably grows in a precipitation range from 600 to 1,300 mm

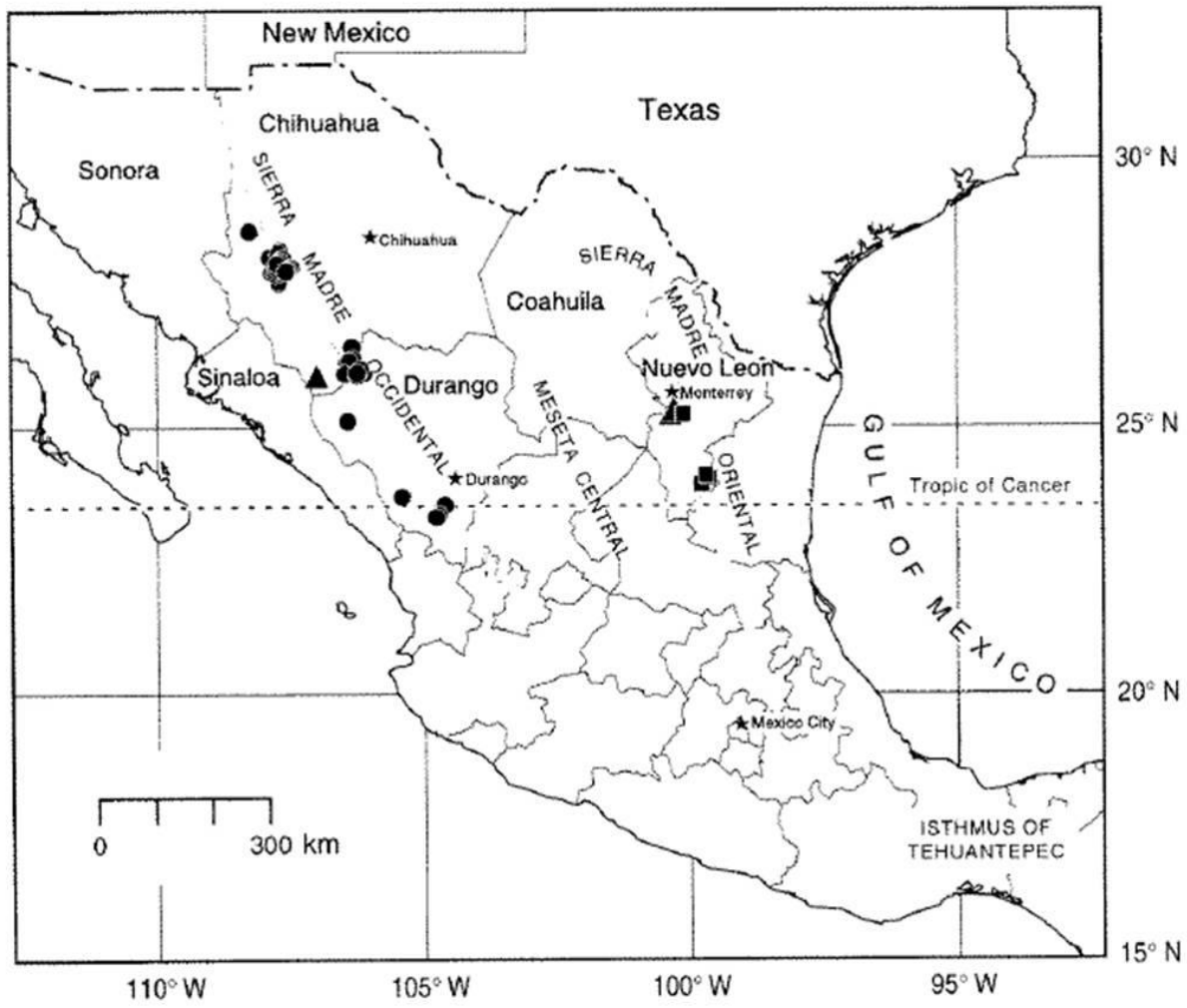

Figure 1. Map of Mexico showing the locations of *Picea chihuahuana* ( ), *Picea martinezii* ( ) and *Picea mexicana* ( ) populations (taken from Ledig et al., 2000a).

considering the estimates obtained using a spline climate model (Sáenz-Romero et al., 2010).

This species is preferentially located in rough terrain areas on hillsides and canyons with slopes ranging from 35 to 80%. The species is also found at the margins of streams and rivers (Narváez, 1984). It is located in north facing areas, showing variations toward northwest and northeast (Narváez, 1984; Sánchez, 1984).

Importance and conservation of *P. chihuahuana* Martínez

Although, the endemic spruces are a minor element in Mexico's flora, they are potentially important from a scientific and conservational point of view. They represent a unique contribution to Mexico's biodiversity and, therefore, have a high value as a genetic resource (Ledig et al., 1997). *P. chihuahuana* occurs in some locations with the highest diversity of tree species at the Sierra Madre Occidental, recognized as a hot spot of species diversity (González-Elizondo et al., 2012). The habitat is a crucial aspect for the protection of this species (Ledig et al., 1997). The discontinuous distribution and variation in population sizes of *P. chihuahuana* provide an excellent opportunity to test the relationships between diversity and population size or degree of isolation (Ledig et al., 1997). Natural reproduction of *P. chihuahuana* has declined, probably by degradation in abiotic conditions, lack of regeneration and high proportion of seed loss caused by pests and inbreeding depression (SARH, 1993; Jacob, 1994; Ledig et al., 1997). Sánchez and Narváez (1990) were the first to propose some alternatives for protection, development and genetic improvement of *P. chihuahuana* by managing the distribution areas of the species, under a concept of botanical reserve.

P. chihuahuana has been used to measure genetic erosion because it is an excellent model for that purpose due to its small and isolated populations (Wehenkel and Sáenz-Romero, 2012). The species has been used to estimate the genetic potential of plant-plant interactions in

Table 1. Some populations of *Picea chihuahuana* in the states of Durango and Chihuahua.

Stand	Ownership	Municipality	Latitude (N)	Longitude (O)	Elevation (m)
Durango					
Arroyo La Pista	Santa María Magdalena de Taxicaringa	Mezquital	23 ° 19'52''	104 ° 45'00''	2,685
Arroyo El Chino	Santa María Magdalena de Taxicaringa	Mezquital	23 ° 24'05''	104 ° 43'05''	2,600
Arroyo Las Lagunas	Private Property	Durango	23 ° 31'24''	104 ° 31'20''	2,775
Arroyo del Infierno	Ejido* El Brillante	Pueblo Nuevo	23 ° 29'40''	105 ° 26'08''	2,725
Faldeo de Cebollitas	Private Property	Canelas	25 ° 05'45''	106 ° 26'35''	2,450
Arroyo de Los Angeles	Ejido El Palomo	Guanaceví	26 ° 00'17''	106 ° 18'14''	2,990
La Estancia-Agua Amarilla	Ejido El Padre y Anexos	Guanaceví	26 ° 00'41''	106 ° 27'13''	2,580
Chihuahua					
Arroyo La Quebrada	Ejido El Caldillo y su anexo El Vergel	Balleza	26 ° 28'12''	106 ° 21'51''	2,730
Río Vinihueachi	Private Property	Bocoyna	27 ° 44'53''	107 ° 41'58''	2,160
El Pinabetal	Comunidad de San Elias	Bocoyna	27 ° 45'42''	107 ° 41'35''	2,305
Las Trojas	Ejido El Ranchito	Bocoyna	27 ° 54'27''	107 ° 45'17''	2,395
Napahuichi I	Private property: La Laja	Bocoyna	27 ° 54'53''	107 ° 37'10''	2,270
Napahuichi II	Private property: La Laja	Bocoyna	27 ° 54'47''	107 ° 37'08''	2,340
Talayote	Ejido Los Volcanes	Bocoyna	27 ° 55'03''	107 ° 49'01''	2,355

Ejido: Land owned and managed by local communities.

natural conditions (Quiñones-Pérez et al., 2014). Finally, *P. chihuahuana* is also of great importance because of its ecological, recreational and aesthetic value and as a refuge for wildlife (Sánchez and Narváez, 1990).

Genetic diversity of *P. chihuahuana* Martínez

Conifers in the family Pinaceae, which includes spruce, are categorized as strongly outcrossing (Schemske and Lande, 1985) with high levels of genetic diversity (Hamrick and Godt, 1989). Population bottlenecks accompanied by inbreeding depression and drift are usually invoked to explain the origin of low levels of genetic diversity. Because the Pinaceae have no self-incompatibility system, selfing is possible when numbers of breeding individuals are reduced, as in refugia or after colonizing events (Ledig et al., 2000b). The (spatial) analysis of genetic diversity in forest species can be estimated by using genetic markers and considering tree positions, the number of variants (polymorphism, polymorphism rate, proportion of polymorphic loci, abundance of genetic variants and average number of alleles or genotypes per locus), or the frequency of variants (effective number of alleles or genotypes, expected heterozygosity, H_e; and Nei's genetic diversity). Genetic diversity studies on the *Pinaceae* family in Mexico have focused on *Pinus,* followed by *Abies* and *Picea*. Thus, the genetic diversity of *P. chihuahuana* has only recently been studied (Ledig et al., 1997; Jaramillo-Correa et al., 2006) (Table 2).

In general, the average expected heterozygosity for *Picea*, estimated by isoenzyme electrophoresis, is H_e= 0.11; the estimated value is below the average limit of genetic variation reported for most gymnosperms (0.17) (Hamrick et al., 1992). Comparing the above results (Table 2) with those reported by Hamrick and Godt (1996), *P. chihuahuana* displays moderate levels of genetic diversity (H_e=0.093) within the observed range for conifers (H_e from 0.055 to 0.131) even within a small geographic area. For example, allozyme analysis used to determine genetic diversity in other conifer species revealed an average H_e of 0.19 (ranging from 0.07 for *Abies guatemalensis* to 0.39 for *Pinus lagunae*) (Aguirre-Plater et al., 2000; Molina-Freaner et al., 2001). These findings suggest the importance of drift and endogamy in the recent evolution of the species (Ledig et al., 1997).

The population's mean heterozygosity of 0.111 expected for the Mexican Martínez spruce is lower than that reported for most other spruces and for conifers in general (Hamrick et al., 1992), but is higher than described for *P. chihuahuana*. The estimated genetic diversity in *P. mexicana* is higher than *P. chihuahuana* and than the average for long-lived, woody endemics, and it is similar to the average for outcrossing endemic plant species in general. This study suggests that P. *mexicana* is a genetically viable species and that threats

Table 2. Genetic diversity reported in some spruce species, including Mexican species.

Specie	AL	P	He	Ho	H cpDNA	H mtDNA	Used Molecular Marker	Reference
Picea abies	-	-	0.371	-	-	-	Allozymes	Lundkvist, 1979
Picea abies	-	-	0.250	-	-	-	RAPD	Jeandroz et al., 2004
Picea abies	-	-	0.545	-	-	-	SNPs	Heuertz et al., 2006
Picea abies	22	-	0.640	0.465	-	-	SSR	Tollefsrud et al., 2009
Picea asperata	10.89	100	0.426	0.400	-	-	SSR	Wang et al., 2004
Picea asperata	1.45	37.1	0.096	0.094	-	-	Allozymes	Luo et al., 2005
Picea asperata	-	-	-	-	0	0.37	mtDNA and cpDNA markers	Du et al., 2009
Picea breweriana	1.48	44.2	0.129	0.121	-	-	Isozymes	Ledig et al., 2005
Picea chihuahuana	1.37	27	0.093	0.073	-	-	Isozymes	Ledig et al., 1997
Picea chihuahuana	-	-	-	-	0.415	0	SSR	Jaramillo-Correa et al., 2006
Picea chihuahuana	-	75	-	-	-	-	AFLP	Wehenkel and Sáenz-Romero, 2012
Picea engelmannii	2.4	80	0.255	-	-	-	Isozymes	Ledig et al., 2006
Picea glauca	3.03	-	0.344	0.342	-	-	Allozyme	O'Connell et al., 2006
Picea glauca	-	-	0.37	-	-	-	STS	Perry and Bousquet, 1998
Picea glauca	1.72	46.8	0.161	0.163	-	-	Allozymes	Godt et al., 2001
Picea glauca	-	-	-	0.52	-	-	SSR	Hodgetts et al., 2001
Picea glauca	10.3	-	0.78	0.71	-	-	SSR	Rungis et al., 2004
Picea glauca	1.92	82	0.345	0.349	-	-	SNPs	Namroud et al., 2012
Picea mariana	2.2	69.2	0.300	0.339	-	-	Allozymes	Isabel et al., 1995
Picea mariana	1.9	82.1	0.321	0.345	-	-	RAPD	Isabel et al., 1995
Picea mariana	2.8	-	-	0.26	-	-	STS	Perry and Bousquet, 1998
Picea mariana	9.5	-	0.72	0.61	-	-	SSR	Rungis et al., 2004
Picea martinezii	1.39	31.9	0.111	0.104	-	-	Isozymes	Ledig et al., 2000b
Picea mexicana	1.4	35.2	0.125	0.136	-	-	Isozymes	Ledig et al., 2002
Picea pungens	1.6	42.7	0.138	-	-	-	Isozymes	Ledig et al., 2006

AL=Number of alleles per locus; P= Percentage of polymorphic loci; He= expected heterozygosity; Ho= observed heterozygosity; H, cpDNA or mtDNA genetic diversity; RAPD= random amplification of polymorphic DNA; SSR= simple sequence repeats (microsatellites); AFLP=amplified fragment length polymorphism; SNPs= single-nucleotide polymorphisms; STS= sequence tagged site.

are primarily environmental (Ledig et al., 2002). *Picea breweriana* is at least as diverse as the endemic spruces of Mexico (*P. chihuahuana*, *P. martínezii,* and *P. mexicana*) (Ledig et al., 2005). Engelmann spruce is much more genetically diverse than the other spruce species. The higher genetic diversity of northern populations of Engelmann spruce may be a result of the wider distribution and larger populations, as well as introgressive hybridization with the transcontinental white spruce; but not in Southwestern populations with evidence of bottlenecks (Ledig et al., 2006). However, the estimated H_e for Engelmann spruce was slightly higher than the average for woody species reported by Hamrick et al. (1992). The diversity of *P. chihuahuana* (*Hcp*), estimated using chloroplast DNA markers, is much higher than that for mtDNA (Jaramillo-Correa et al., 2006), probably because of a much more active and wider gene flow by pollen dispersion than by seed dispersion. The latter authors found that none of the 16 *P. chihuahuana* populations surveyed was polymorphic for the mtDNA markers, while for cpDNA markers, 3 of the 16 stands surveyed were fixed for a particular chlorotype (Table 2).

In one study of *P. chihuahuana*, the degree of genetic erosion was evaluated in five populations located in the State of Durango (Figure 2) by comparing the genetic diversity using diameter classes. For this study, AFLP (Amplified Fragment Length Polymorphism) molecular markers were used and genetic diversity was quantified (δ_t, Gregorius total differentiation were 0.183, 0.162, 0.150, 0.117 and 0.136, for each population, respectively as shown in Figure 2) (Gregorius, 1987; Wehenkel and Sáenz-Romero, 2012). The results revealed significant genetic erosion only for the smallest population, San José de las Causas (Wehenkel and Sáenz-Romero,

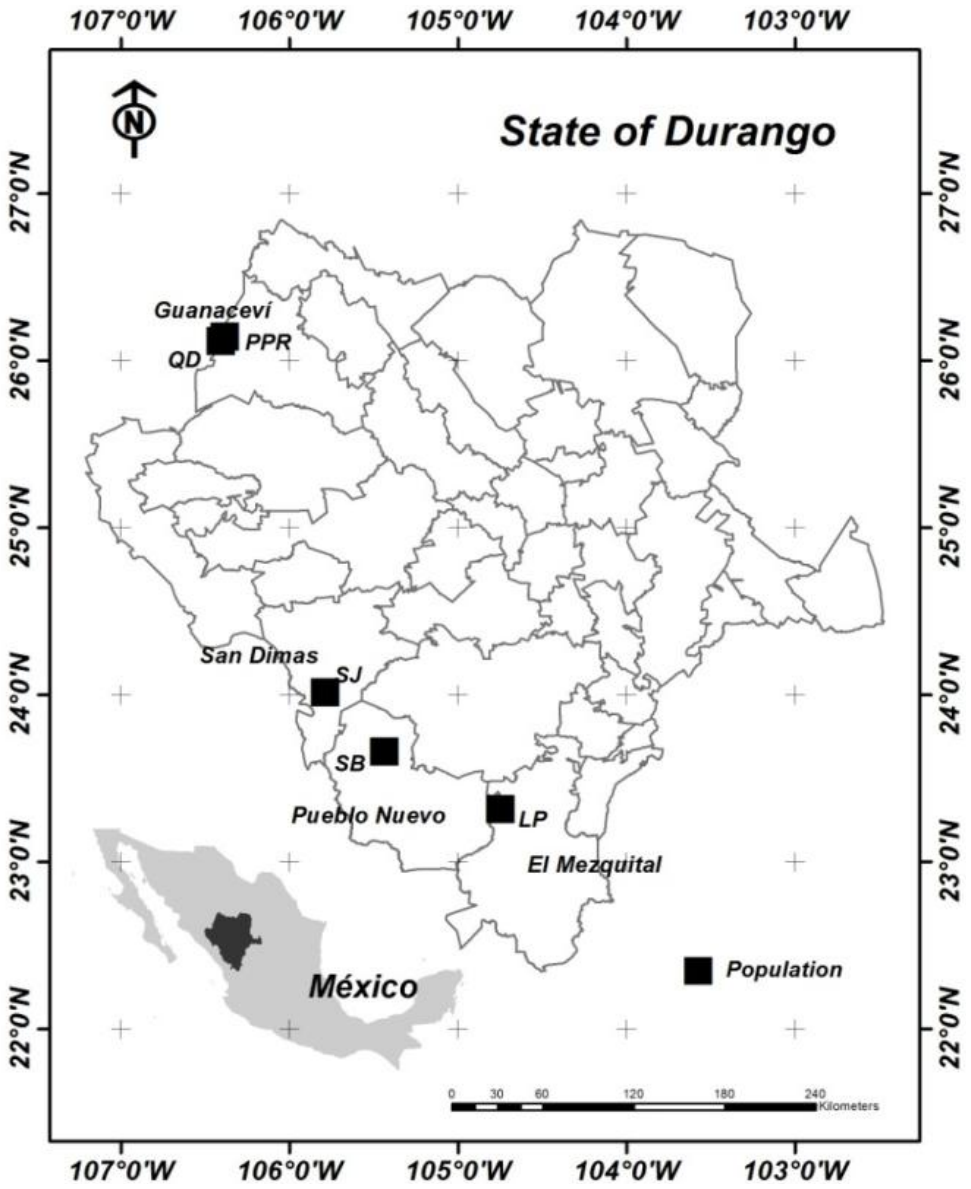

Figure 2. Map of the five study populations of *Picea chihuahuana* Martínez located in the State of Durango, Mexico: Paraje Piedra Rayada (PPR), Quebrada de los Durán (Arroyo del Indio Ignacio) (QD), La Pista (LP), Santa Bárbara (Arroyo del Infierno) (SB) and San José de las Causas (SJ) (black boxes) (taken from Quiñones-Pérez et al., 2014).

2012). Therefore, if genetic diversity determined by AFLP loci reflects diversity in the whole genome, genetic erosion *per se* does not explain fragmentation and restricted distribution of *P. chihuahuana,* except in the smallest populations. In this case, genetic diversity would not be sufficient to prevent depression due to endogamy; also mutations could not compensate the loss of alleles as a result of genetic drift (Wright, 1938; Millar and Libby, 1991; Frankham et al., 2002; Bücking, 2003; Wehenkel and Sáenz-Romero, 2012).

In another study (Quiñones-Pérez et al., 2014), interactions between AFLP variants of *P. chihuahuana* and its two most dominant nearest-neighboring tree species were analyzed in five populations located in Durango, Mexico (Figure 2). The results show a statistically significant association between such genetic variants - at one AFLP locus in one *Picea* population - and two nearest-neighbor conifer species (*Picea chihuahuana* Martínez and *Pinus arizonica* Engelm.). The findings indicate that interactions between genetic variants and species diversity may be crucial in shaping plant communities. They also indicate the existence of interactions between different levels of diversity, e.g., between specific genetic variants of a single species and neighboring tree species. However, further studies are required for a better understanding of the possible roles that such asso-

ciations between genetic variants might play (Quiñones-Pérez et al., 2014).

Perspectives for management and conservation of *P. chihuahuana* Martínez

The maintenance of genetic variation is considered essential for long-term survival of the species. Consequently, the rate of loss of genetic diversity in isolated populations is a great concern for conservation and protection of rare or endangered species (Ledig et al., 1997). It is worth mentioning that small populations are more susceptible to extinction due to various factors such as demographic stochasticity, as well as environmental, genetic and natural catastrophes (Shaffer, 1981); this may apply to some *P. chihuahuana* populations. Therefore, the concept of minimum viable population (MVP) is appropriate, and it is widely applied for recovery and conservation management programs; this concept is also relevant in the IUCN Red List criteria for small and restrictted populations (Traill et al., 2011). Thus, MVP is frequently defined as the smallest population showing a 95 to 99% chance of persistence in a given time period (Ewens et al., 1987). Calculation of the MVP allows the planning of management strategies for conservation of endangered species, as the case of Mexican spruces including *P. chihuahuana* (Mendoza-Maya et al., submitted). In a meta-analysis involving different species, the reported size of the minimum viable population was on average 4,169 individuals (Traill et al., 2011). Therefore, all populations, including *P. chihuahuana,* are unlikely to survive without help, because according to Ledig et al. (2000), the maximum size of a population of this species is only 3,564 trees (> 2 m in height) (Wehenkel and Sáenz-Romero, 2012).

Recent studies have shown that climate change will affect rare species such as *P. chihuahuana*. Therefore, the populations could be relocated by assisted colonization (Ledig et al., 2010; Ledig, 2012) to sites where it is predicted that the climate to which the populations are adapted will occur. Assisted colonization (also named assisted migration) need to be done at present, targeting sites will occur in suitable climate by year 2030 or so. That is, in order to have alive adult trees in the future, realigned to the climate for which they are adapted, aiming that those trees would be enough healthy to produce viable seed and thus they will be able to produce a new generation (that eventually might need to be relocated again, if climatic change would continue as predicted). Assisted colonization or migration need target a climate projection not too far away in time, because at present seedlings would be killed by frost damage (Loya-Rebollar et al., 2013). Although it could be argued that natural communities recipients of the population relocated could be altered, there are no reason to believe that natural plant communities will continue unaltered by climatic change. Worldwide evidence is emerging now of forest decline, sometimes massive, induced by climatic change (Allen et al., 2010; Mátyás, 2010; Alfaro et al., 2014). Thus, in our view, inaction imply larger risks than a proactive realignment of the populations to the climate for which they are adapted. Therefore, active conservation programs must be implemented to preserve *P. chihuahuana*. In a recent study, a management proposal for *in situ* and *ex situ* conservation of three *Picea* species from Mexico, including *P. chihuahuana*, was presented (Mendoza-Maya et al., submitted). This was based on the diversity and genetic structure of populations, as well as on projected climate scenarios for 2030, 2060 and 2090. Thus, the protection of eight *P. chihuahuana* populations, determined from the formula N_e= -984.58 + (36723 H_e) (Viveros-Viveros et al., 2010), was proposed for *in situ* and *ex situ* conservation. There are some indications of a requirement for a genetically effective population size (Millar and Libby, 1991) of 2,541 individuals to conserve a given average heterozygosity (H_e= 0.096) in *P. chihuahuana* populations in the state of Chihuahua (Mendoza-Maya et al., submitted).

Some of the following actions could increase the population size: 1) *ex situ* conservation along with assisted migration in response to climate change, 2) increasing the size of the smallest populations by propagating individuals, 3) increasing the genetic diversity to restrain endogamy and to prevent the presence of extinction vortex, 4) protection of natural regeneration by acting against livestock, wildlife and forest fires, 5) establishment of artificial regeneration using reproductive local material gathered from well-selected sites in the population's boundaries (but not within the population), and 6) elimination of competing vegetation (other tree species) in the vicinity of the particular population. Finally, continuous monitoring of size and genetic diversity of the current populations *in situ* is essential (Wehenkel and Sáenz-Romero, 2012).

Efforts should focus on the protection of populations displaying the highest degree of genetic variation because these populations have the greatest potential for adaptive evolution (Ledig, 2012).

Conflict of Interests

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

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Appraisal of biochemical and genetic diversity of mango cultivars using molecular markers

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Mango (*Mangifera indica* L.) is one of the oldest fruit crops and is broadly cultivated worldwide. To determine the level of genetic diversity, a total of 13 mango genotypes have been collected from different farms of Fayoum oasis in Egypt and were analyzed using molecular (DNA) and biochemical (SDS-PAGE) markers along with the quantification of soluble carbohydrates, chlorophyll and carotenoids. These profiles were evaluated as characters to identify the taxonomic relationships of these genotypes. A total of 433 protein bands (ranged from 8 to 180 KDa) from all genotypes, were detected in SDS-PAGE. A total of 306 RAPD fragments were produced by 19 primers and among them 123 (40.2%) were polymorphic. The similarities between different taxa were estimated by Jaccard's similarity index and clustered in neighbour joining clustering tree. Among the 13 tested mango samples, the total carbohydrate contents ranged between 31.9 and 40.8 µg/100 mg fresh weights, which represents Taymour cultivar and accession No. 7, respectively. Of the 13 mango cultivars and accessions studied, the highest chlorophyll content (386.9 µg/g) was found in accession No. 10; whereas, the lowest value was observed with accession No. 12 (202.5 µg/g). The amounts of carotenoids were wide-ranging and reached a maximum value of 106.2 µg/g with accession No. 9, however, accession No. 8 recorded the lowest concentration (19.9 µg/g). In conclusion, RAPD-PCR and SDS-PAGE were proved to be an efficient tool in assessing the genetic diversity of mango genotypes. It will also provide an important input to breeders for mango improvement program.

Key words: Mango, genetic diversity, chlorophyll, SDS-PAGE, RAPD.

INTRODUCTION

Mango (*Mangifera indica* L.) is commonly known as the 'king of fruits'. A native of Southeast Asia is one of the important fruit crops in the tropical and subtropical regions thought to have been introduced to Africa in the

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Abbreviations: SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; **RAPD,** random amplified polymorphic DNA.

14th century (Singh, 1960; Duval et al., 2005). Most cultivated mangoes belong to the species *Mangifera indica*; one of 600 species in the family *Anacardiaceae*. The genus *Mangifera* contains around 70 species, which can be divided into two subgenera, Limus and Mangifera (Kostermans and Bompard, 1993) with at least 26 species producing edible fruits (Mukherjee, 1997; Tanaka, 1976). Mango plays a major role in the global trade as it constitutes approximately 50% of all tropical fruits produced worldwide, equivalent to 5.5% of all fruit produced globally (Jedele et al., 2003; Vasanthaiah et al., 2007). In Egypt, mango is an important fruit crop and according to the latest statistics provided by the Ministry of Agriculture and Land Reclamation of Egypt (2007), a total of 184204 Feddan are cultivated with mango.

Mango has economic as well as therapeutic value due to its high vitamin, mineral and fiber content (Lakshminarayana, 1980). Ripe fruits are freshly eaten or prepared as juice, jams, jellies, and squash or canned, while unripe fruits are used in pickles, chutneys and other culinary arrangements. The various parts of mango are used as antihelmintic, diaphoretic, and refrigerant agents and in bleeding piles, manorrhagia, scabies and cutaneous infections, leucorrhoea, diarrhea and dysentery (Lakshminarayana, 1980; Mukherjee, 1997).

Genetic diversity is one of the key factors for the improvement of many crop plants including mango. Plant breeders rely on the availability of genetic diversity during selection in cultivar development.

It has been proposed that traditional agro-ecosystems maintain a high diversity of cultivated plants, both in terms of crop species and genotypes within each species (Alcorn, 1981; Altieri et al., 1987; Bellon, 1996; Brush, 1989, 1992, 2000). Human action, as well as ecological and evolutionary processes, promotes the maintenance of genetic variation of crops within traditional agro ecosystems (Lambert, 1996).

Varieties developed with wider genetic base may be helpful in enhancing the yield under various agro-climatic conditions (Asif et al., 2005). Diverse genetic base may also resist the spread of diseases (Zhu et al., 2000) in approved varieties. The study of genetic diversity is also important for varietal identification, proper purity maintenance, for the implementation of plant variety protection rights and export under WTO regulations. Mango has been reported to have extensive diversity due to continuous grafting and phenotypic differences arising from varied agro climatic conditions in different areas (Young and Ledin, 1954; Ravishankar et al., 2000).

Genetic diversity can be accessed from pedigree analysis, morphological traits or using molecular markers (Pejic et al., 1998).

A number of reports are available on the use for DNA markers to assess genetic diversity among species of several horticultural crops, as well as validation of genetic relatedness among them (Bhat et al., 2010).

Molecular markers have diverse applications in crop improvement, particularly in the areas of genetic diversity and varietal identification studies, gene tagging, disease diagnostics, pedigree analysis, hybrid detection, sex differentiation and marker assisted selection. DNA markers can be used to diagnose the presence of the gene without having to wait for gene effect to be seen (Bhat et al., 2010; Botez et al., 2009; Sisko et al., 2009; Thimmappaiah et al., 2009).

Molecular markers are useful tools for estimating genetic diversity as these are not influenced by environment, are abundant and do not require previous pedigree information (Bohn et al., 1999). Among the biochemical markers, SDS-PAGE has been widely used due to its simplicity and effectiveness for estimating genetic diversity.

Among the different DNA marker types, random amplified polymorphic DNA (RAPD) markers have frequently been used for genetic analyses (Langridge et al., 2001) due to simplicity, efficiency and non requirement of sequence information. RAPDs assay detects nucleotides sequence of polymorphisms in DNA using only a single primer pair of arbitrary nucleotide sequence (Welsh and McClelland, 1990; Williams et al., 1990). RAPDs have been widely used for identification of genotypes in crop plants, for investigating the genetic variability within species and to show relationships among populations (Freitas et al., 2000).

Like all living organisms, plants require energy in chemical form so they can grow and carry out basic life functions. Plants produce, store and burn carbohydrates in the form of sugar to provide them energy (Bieleski, 1962; Bieleski et al., 1992). Carbohydrates are the most abundant single class of organic substances found in nature and initially synthesized in plants from a complex series of reactions involving photosynthesis (Giaquinta, 1979; Gayler and Glasziou, 1972).

There are many pigments in the higher plants, such as chlorophyll, carotenoids, phtochrome, flavonoid, anthocyanin, tannin, and many others (Yang et al., 1998). Chlorophyll is an extremely important bio-molecule that absorbs sunlight and uses its energy to synthesize carbohydrates from CO_2 and water. This process is known as photosynthesis and is the basis for sustaining the life processes of all plants (Blachburn, 1998). Plants contain both chlorophyll a and chlorophyll b, which have slightly different structures (Carter and Spiering, 2002). Carotenoids are composed of two small six-carbon rings connected by a "chain" of carbon atoms. Carotenoids act as accessory pigments, harvesting light for photosynthesis and as photo protective agents limiting the damaging effects of high irradiance (Johnson et al., 1993; Feruse and Arkosiova, 2001). Carotenoids protect cells and tissues from free radicals and also function as light collectors (Blachburn, 1998). During recent years there has been remarkable progress in chlorophyll and carotenoids quantification as an intriguing tool that can reveal information on plant performance and cultivars relationship (Kulshreshtha et al., 1987).

Table 1. List of mango cultivars and accessions used in the current study.

Code	Variety and status	Location
1	Alphons (commercial cultivar)	Fayoum oasis
2	Taymour (commercial cultivar)	Fayoum oasis
3	Ewais (commercial cultivar)	Fayoum oasis
4	Zebda (commercial cultivar)	Fayoum oasis
5	Mabrouka (commercial cultivar)	Fayoum oasis
6	Local cultivar (accession)	Fayoum oasis
7	Local cultivar (accession)	Fayoum oasis
8	Local cultivar (accession)	Fayoum oasis
9	Local cultivar (accession)	Fayoum oasis
10	Local cultivar (accession)	Fayoum oasis
11	Local cultivar (accession)	Fayoum oasis
12	Local cultivar (accession)	Fayoum oasis
13	Local cultivar (accession)	Fayoum oasis

Table 2. Primer sequences examined for categorization of mango cultivars and accessions.

Primer code	Primer Sequence (5' → 3')	Primer code	Primer Sequence (5' → 3')
OP-A01	CAGGCCCTTC	OP-B16	TTTGCCCGGA
OP-A02	TGCCGAGCTG	OP-B18	CCACAGCAGT
OP-A04	AATCGGGCTG	OP-B20	GGACCCTTAC
OP-A05	AGGGGTCTTG	OP-C01	TTCGAGCCAG
OP-A09	GGGTAACGCC	RMn-P1	CAGAAGCGGA
OP-A11	CAATCGCCGT	RMn-P2	GGGTAACGCC
OP-A16	AGCCAGCGAA	RMn-P3	TGTCATCCCC
OP-A18	AGGTGACCGT	RMn-P4	AAGTGCGACC
OP-A20	GACCAATGCC	RMn-P5	ACTGAACGCC
OP-B15	GGAGGGTGTT		

The objective of the present study was to assess the level of genetic diversity in the gene pool of mango using RAPD and SDS-PAGE with the quantification of soluble carbohydrates, leaf chlorophyll and carotenoids.

MATERIALS AND METHODS

Plant materials

The experimental materials for the present study comprised of 13 local and commercial Egyptian mango cultivars and accessions collected from different farms of Fayoum oasis in Egypt. The taxonomy and classification of the used samples were identified according to Hussein (2009) as demonstrated in Table 1.

SDS-PAGE analysis

To study the protein banding pattern of 13 mango genotypes, we used SDS-PAGE, which was performed according to the method of Laemmli (1970), as modified by Studier (1973). Total proteins were extracted from mango trees leaves. Protein fractionations were performed exclusively on vertical slab (19.8 × 26.8 × 0.2 cm) gel using the electrophoresis apparatus manufactured by Laboconco. The Blueye Prestained Protein Ladder (GeneDirex) was used as a standard marker. The bands were detected and analyzed using Total Lab software.

DNA extraction, primers and DNA amplification

Genomic DNA was extracted from small amount of young and fresh leaves (0.1 g) from the 13 cultivars and accessions of mango trees by the Biospain Plant Genomic DNA Extraction Kit (BioFlux).

Nineteen primers (Table 2), obtained from Pharmacia Biotech. (Amersham Pharmacia Biotech UK Limited, Ebgland HP79 NA), were used for identification of different mango genotypes.

PCR reaction test was performed in a 25 µL volume reaction mixture containing: 5 µL of 5X green Taq DNA polymerase buffer, 100 µM of primer (1 µL), 20 ng of total cellular DNA, 10 mM of each dNTP (0.5 µL), 25 mM $MgCl_2$ (4 µL), 5 U/µL of GoTaq DNA polymerase (0.25 µL) (Promega), up to 25 µL by nuclease-free water. PCR was performed in a DNA thermo cycler (Biometra, Germany). Samples were first heated at 94°C for 3 min and subjected to 35 cycles of the following cycle: 45 s at 94°C, 45 s at 37°C, 1.5 min at 72°C. A

Table 3. Total soluble carbohydrates (µg/100mg), total chlorophyll (µg/g), and carotenoids content (µg/g) in different genotypes of mango trees (mean ± SE).

Code	Total soluble carbohydrates µg/100mg)	Total chlorophyll (µg/g)	Carotenoids (µg/g)
1	35.5 ±3.79	210.9 ± 14.5	28.0 ± 2.43
2	31.9 ±4.04	205.2 ±11.78	29.4 ±2.56
3	38.5 ±2.45	273.2 ±21.2	40.9 ±3.5
4	37.2 ±3.39	272.9 ±18.7	30.9 ±1.51
5	34.6 ±2. 9	323.3 ±24.8	99.9 ±4.81
6	32.6 ±3.97	366.9 ±22.91	54.8 ±2.47
7	40.8 ±3.54	378 ± 21.1	66.8 ±4.69
8	32.4 ±4.13	276.3 ±15.9	19.9 ±1.23
9	39.8 ±3.93	331.9 ±20.5	106.2 ±7.17
10	35.4 ±3.28	386.9 ±23.8	37.3 ± 2.61
11	38.4 ± 4.1	318.4 ±27.58	48.8 ±2.48
12	36.3 ±3.97	202.5 ±13.52	28.3 ±1.92
13	32.2 ±3.58	322 ±20.31	47.0 ±2.57

A final step of 5 min at 72°C was always run. PCR reaction was tested on 1.6% agarose (Genetics) gels and 100 bp DNA ladder H3 RTU (Genetics) was used as the standard marker.

Total soluble carbohydrates estimation

Total soluble sugars content were assayed at 625 nm using anthrone reagent (Cao et al., 2007).

Total chlorophyll and carotenoids analysis

Total chlorophyll (The sum of chlorophyll a and b) and total carotenoids (Car) concentrations were determined following the methods of Porra et al. (1989) and Lichtenthaler (1987), respectively on the basis of µg /g fresh weight according to the following formula:

Total chlorophyll (TC) = $17.76A_{646.6} + 7.34A_{663.6}$ (µg/ml)
Total carotenoids = $4.69A_{440.5} – 0.267$ x TC (µg/ml)

Data handling and cluster analysis

The size of DNA fragments and protein band molecular weights were calculated using Total Lab software. Data were scored for computer analysis on the basis of the presence (1) or absence (0) of the amplified products for each primer. Pair wise comparisons of genotypes, based on the presence or absence of unique and shared polymorphic products, were used to determine similarity index, according to Jaccard (1980). The similarity coefficients were, then, used to construct dendrogram, using Neighbour joining cluster algorithm employing the Paleontological Statistics (PAST) software Version 2.17b (Hammer et al., 2001).

RESULTS AND DISCUSSION

Total carbohydrate

Data in Table 2 shows the total carbohydrates did not differ much within the tested 13 mango genotypes. Taymor cultivar showed the lowest level of total carbohydrates (31.9 µg/100 mg) while, the highest (40.8 µg/100 mg) was found in accessions No. 7.

Reid et al. (1989) have reported that decapitation and girdling of *Protea* plant flowers slow down the leaf blackening and he proposed that reduced carbohydrate content was the cause of that phenomena. Bieleski et al. (1992) reported that the changes in carbohydrate content of *P. eximia* leaves are consistent with our hypothesis that the leaf-blackening disorder arises out of a depletion of leaf carbohydrate. Leaf blackening was visible shortly after the starch and sugar content of the leaves had fallen to their minimum level. Moreover, Boldingh et al. (2000) reported that glucose peak in early development of *A. deliciosa* coincides with a peaks of water content. Transient accumulation of sugars at the early stage of growth may lead to an increase in osmotic pressure followed by an increase in cell turgor at the expansion phase.

Total chlorophyll and carotenoids determination

Data presented in Table 3 illustrate the total chlorophyll content (Chl a+b), in leaves of 13 different mango genotypes which ranged between 202.5 to 386.9 µg/g fresh weight. It is clear that, the highest chlorophyll content (386.9 µg/g) was found in accession No. 10, whereas the lowest value was observed with accession No. 12 (202.5 µg/g). Carotenoids had significantly varied results, the concentrations were in a wide scale and ranged from 19.9 to 106.2 µg/g, the accessions No. 9 shows the highest concentration (106.2 µg/g), however, accessions No. 8 recorded the lowest concentration (19.9 µg/g). These results are in agreement with Kershaw and Webber (1986) who examined seasonal changes in chlorophyll concentrations and photosynthetic rates in *Brachythecium*

rutabulum from an apple orchard in Canada. Samples collected from a low-light environment, exhibited higher chlorophyll concentrations and higher rates of photosynthesis at low light, relative to samples collected when understory irradiance was greater. So, it was clear that the variation in pigments concentrations among different genotypes maybe a reason of genetical or environmental variance.

Molecular and biochemical identification of some genotypes of mango

Genome profiling is a strategy that identifies genomic DNA fragments common to closely related species without performing DNA sequencing. Random RAPD-PCR is one of the key technologies of genome profiling (Naimuddin et al., 2002). PCR based methods including RAPD can be effectively used for cultivars identification and the study of phylogeny and genetic diversity (Saengprajak and Saensouk, 2012).

Initial evaluating of 19 RAPD primers against 13 cultivars and accessions of mango trees gave 123 polymorphic bands and 183 fragments were monomorphic between the plant genotypes (Figure 1 and Table 4). The total 306 bands were amplified using 19 RAPD primers; produced an average of 7 (OP-B16) to 24 (Mnp3) bands per primer. The number of RAPD fragments that were amplified ranged from 1 (OP-A01) to 19 (Mnp5) with an average of 16.1 bands per primer and the sizes ranged from about 65 to 2100 bp. However, the highest number of DNA amplified fragments, using the 19 primers, was present in cultivar Taymour (Table 5) (255 fragments), while the accession No. 13 revealed the lowest number (227 fragments).

For the polymorphism percentage presented by the 19 primers which used to identify the genotypes as shown in Table 4, the primer OP-B18 gave the highest number of polymorphic fragments in all genotypes (18 fragments) with 79.3% polymorphism percentage while the primers OP-B15 and OP-B20 gave the lowest number of polymorphic fragments (1 fragment) with 7.1 and 9.1%, respectively of polymorphism percentage.

As showed in Table 6, the band data were utilized to calculate the similarity matrix. Jaccard's similarity coefficient ranged from 0.755 (between the accessions 6 and 13) to 0.893 (accessions 6 and 7). The RAPD results revealed a large set of markers, which can be used for the evaluation of both between- and within-species genetic variation (Guadagnuolo et al., 2001).

Figure 2 illustrate the neighbour joining clustering tree which was constructed on the basis of Jaccard's coefficient based on RAPD-PCR. The tree clustered the cultivars and accessions into two clusters (I and II). The clusters I divided to two groups (A and B). The group A contained most of the accessions (7, 6, 8, 9, 13, 11 and 10) while the group B included the cultivars Taymour and alphons and only one accession No. 12. However, the cluster II consisted of three cultivars which are Mabrouka, Ewais and Zebda.

On the other hand, to identify the 13 genotypes of mango, we used another method which is SDS-PAGE technique. The banding patterns were analyzed on 12% SDS-PAGE. Two bands corresponding to 18.5, 17 and 16 KDa were observed in 12% SDSPAGE (Figure 3).

The 18.5 KDa band was reported in all cultivar genotypes Alphons, Taymour, Ewais, Zebda and Mabrouka but it disappeared in all the accession genotypes. 17 KDa band was detected in cultivars alphons, Taymour, Ewais and Zebda and in the accessions 6, 10, 11, 12 and 13. Moreover, the 16 KDa band appeared in Mabrouka and in the accessions 7 and 8. That is in agreement with the results of Zaied et al. (2007) who found variation between different genotypes of mango at SDS-PAGE level. The similarity between the cultivars and the accession was high which ranged from 91.4 to 100% (Table 7).

Mabrouka cultivar and the accessions 6, 10, 11, 12 and 13 showed 91.4% of similarity. Also, the similarity between the accessions 7 and 8 and four cultivars Alphons, Taymour, Ewais and Zebda was 91.4%. However, the similarity rose up to 100% between 13, 6, 10, 11 and 12 and among 7 and 8. Moreover, the cultivars Alphons, Taymour, Ewais and Zebda revealed the highest similarity (100%). Neighbour joining clustering based on SDS-PAGE results showed that the cultivars and the accessions under study were set in two clusters (I and II). The cluster I contained two groups (A and B). Group A include accessions 8, 7, Mabrouka and accession 9, while group B contain Zebda, Taymour, Ewais and Alphons cultivars (Figure 2). Furthermore, the cluster II consisted of the accessions 6, 13, 12, 11 and 10. Some of the accessions grouped together either from RAPD-PCR results or from SDS-PAGE, and that could refer to their common ancestor which may be one of the cultivars under study or another cultivar was not used in our study. Our results show that RAPD-PCR and SDS-PAGE are useful for taxonomy and evaluation study between different cultivars and accessions of mango. That is in agreement with the study of Ghafoor and Arshad, (2008) that reported that the electrophoretic patterns of total proteins (SDS-PAGE) have been successfully employed to resolve the taxonomic and evolutionary problems of some plant species. The use of RAPD technique for the study of genetic variation has been demonstrated as suitable in many species (Abbas et al. 2009). Moreover, we can report that the RAPD-PCR and SDS-PAGE results indicated existence variations between these genotypes. The molecular and/or biochemical methods are more powerful than morphological traits to study the genetic diversity. Ungerer et al. (2003) and Alan (2007) showed that the estimation of genetic diversity based on the morphological traits alone showed the true level of genetic diversity between genotypes because morphological traits are determined by the interaction between genetic

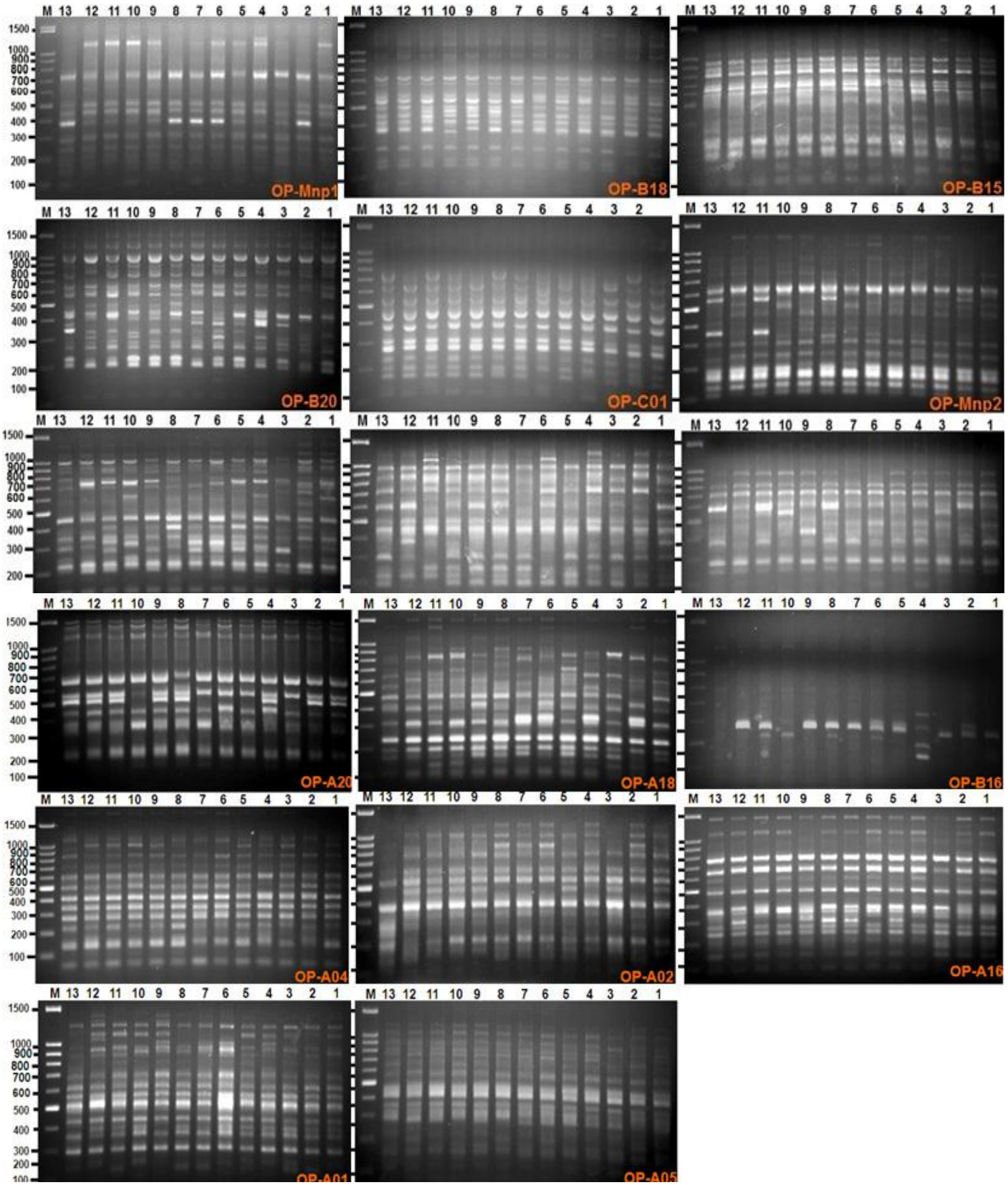

Figure 1. DNA amplified fragments using random primers (OP- Mnp1, B18, B15, B20, Co1, Mnp2, Mnp3, Mnp4, Mnp5, A20, A18, B16, A04, A02, A16, A01 and A05). Commercial cultivars: (1) Alphons, (2) Taymour, (3) Ewais, (4) Zebda and (5) Mabrouka. Accessions (6, 7, 8, 9, 10, 11, 12 and 13) uses as a stock. M: 1Kb markers.

Table 4. Genetic polymorphism between five cultivars (1) Alphons, (2) Taymour, (3) Ewais, (4) Zebda and (5) Mabrouka and eight accessions 6, 7, 8, 9, 10, 11, 12 and 13 of mango detected by RAPD-PCR.

Primer	Size of fragments (bp)	Total bands	Number of monomorphic bands	Number of polymorphic bands	Polymorphism percentage (%)
OP-A01	240-1390	19	8	11	57.9
OP-A02	144-1240	15	8	7	46.7
OP-A04	65-2100	14	12	2	14.3
OP-A05	125-1120	18	16	2	11.1
OP-A09	130-1240	12	8	4	33.3
OP-A11	120-1370	16	9	7	43.8
OP-A16	110-1490	20	13	7	35.0
OP-A18	110-1520	23	5	18	78.3
OP-A20	240-1520	11	10	1	9.1
OP-B15	150-1065	14	13	1	7.1
OP-B16	440-1170	7	1	6	85.7
OP-B18	180-1120	16	14	2	12.5
OP-B20	210-1450	24	11	13	54.2
OP-C01	125-725	13	10	3	23.1
Mnp1	150-1240	12	9	3	25.0
Mnp2	110-1240	20	11	9	45,0
Mnp3	140-1270	24	7	17	70.8
Mnp4	190-1250	15	7	8	53.3
Mnp5	125-975	13	11	2	15.4
Total	-	306	183	123	40.2

Table 5. Total bands produced from each primer for the five cultivars (1) Alphons, (2) Taymour, (3) Ewais, (4) Zebda and (5) Mabrouka (1) Alphons, (2) Taymour, (3) Ewais, (4) Zebda and (5) Mabrouka and eight accessions 6, 7, 8, 9, 10, 11, 12 and 13 of mango.

Primers	13	12	11	10	9	8	7	6	5	4	3	2	1
OP-A01	11	17	12	13	14	13	12	14	14	15	14	14	14
OP-A02	7	11	10	10	11	12	12	12	11	10	9	12	11
OP-A04	13	14	14	13	14	12	12	12	13	14	13	13	13
OP-A05	17	17	17	17	17	17	17	17	17	17	17	17	17
OP-A09	10	9	10	9	8	9	10	10	9	9	9	10	9
OP-A11	11	12	12	12	14	14	12	12	14	12	12	12	12
OP-A16	15	16	15	14	13	15	16	15	15	16	16	17	15
OP-A18	14	14	18	20	17	19	15	19	18	14	13	17	13
OP-A20	11	11	11	10	11	11	10	10	10	11	10	11	11
OP-B15	14	14	14	14	14	14	14	14	14	14	14	14	13
OP-B16	1	3	3	2	2	3	2	4	3	5	3	3	3
OP-B18	14	16	16	16	15	16	16	15	14	14	15	15	15
OP-B20	16	19	18	20	19	18	19	21	18	18	19	19	19
OP-C01	11	11	11	12	12	13	12	12	12	12	12	11	11
Mnp1	9	11	10	10	10	9	10	10	10	11	10	9	10
Mnp2	14	14	15	14	15	17	16	17	14	16	14	15	15
Mnp3	15	18	17	16	15	15	15	16	17	17	13	20	18
Mnp4	11	11	11	11	10	10	10	13	9	12	11	13	12
Mnp5	13	13	13	13	12	13	11	11	13	12	13	13	13
Total	227	251	247	246	243	250	241	254	245	249	237	255	244

Table 6. Genetic similarity matrix detected between five cultivars (1) Alphons, (2) Taymour, (3) Ewais, (4) Zebda and (5) Mabrouka and eight accessions 6, 7, 8, 9, 10, 11, 12 and 13 of mango with RAPD markers based on Jaccard's coefficients.

Genotype	13	12	11	10	9	8	7	6	5	4	3	2	1
13	1												
12	0.781	1.000											
11	0.823	0.855	1.000										
10	0.775	0.862	0.871	1.000									
9	0.787	0.854	0.856	0.849	1.000								
8	0.800	0.832	0.875	0.848	0.867	1.000							
7	0.789	0.816	0.817	0.831	0.851	0.877	1.000						
6	0.755	0.820	0.822	0.856	0.827	0.860	0.893	1.000					
5	0.785	0.865	0.860	0.867	0.866	0.865	0.848	0.845	1.000				
4	0.770	0.828	0.797	0.830	0.822	0.808	0.804	0.822	0.833	1.000			
3	0.761	0.842	0.837	0.851	0.802	0.821	0.817	0.809	0.883	0.824	1.000		
2	0.779	0.850	0.846	0.839	0.798	0.870	0.820	0.831	0.815	0.806	0.826	1.000	
1	0.772	0.879	0.860	0.896	0.818	0.844	0.813	0.832	0.864	0.840	0.876	0.876	1.000

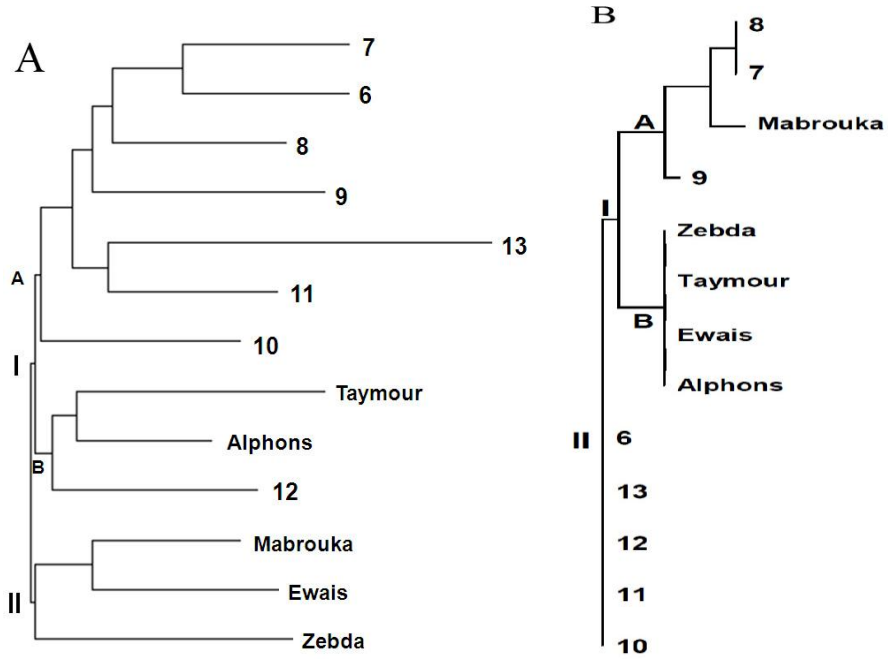

Figure 2. (A) RAPD based dendogram of the thirteen cultivars and accessions constructed using Neighbour joining method based on Jaccard's coefficient. **(B)** Dendogram showing the similarity among the electrophoretic protein patterns (SDS-PAGE) of 13 of cultivars and accessions based on Jaccard's similarity coefficient values which were grouped by the Neighbour joining method.

and environmental factors. Gene expression is influenced by the environment therefore; the selection based merely on morphological traits has been often misleading (Kumar et al., 1998; Astarini et al., 2004 and Asif et al., 2005). In fact RAPD-PCR is a useful technique for providing information on the degree of polymorphism and genetic diversity of our cultivars and accessions. So this analysis could be profitable for breeders for rapid and early identification of most diverse genotypes to improve crop productivity. The knowledge of the genetic diversity of the genotypes is important for parental selection that to maximize the genetic improvement. But we found that RAPD-PCR technique was much higher and an efficient method than that of the SDS-PAGE for genotypes identification because the RAPD-PCR markers are stable but the markers appearing in SDS-PAGE could be affected by

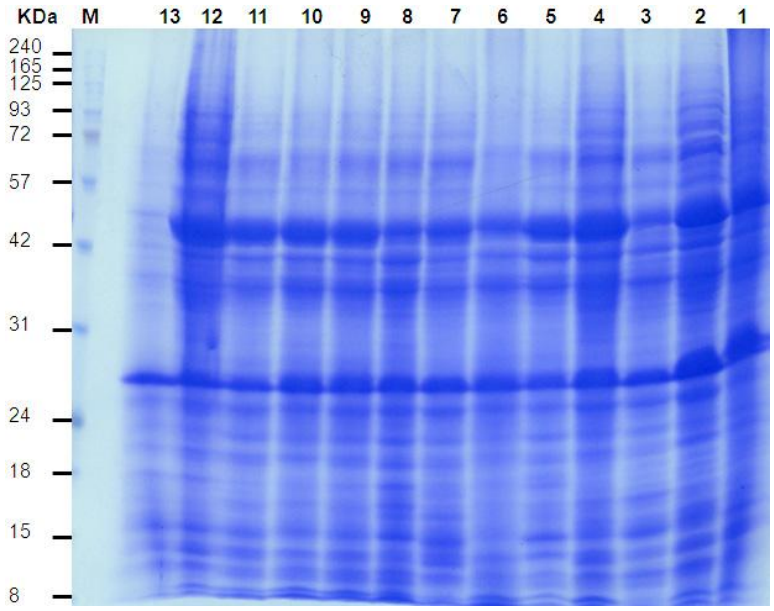

Figure 3. SDS-PAGE banding patterns of 13 cultivars and accessions of mango. Cultivars: (1) Alphons, (2) Taymour, (3) Ewais, (4) Zebda and (5) Mabrouka. Accessions: 6, 7, 8, 9, 10, 11, 12 and 13. Wild type varieties used as a stock M: Protein marker.

Table 7. Genetic similarity matrix appeared between five cultivars (1) Alphons, (2) Taymour, (3) Ewais, (4) Zebda and (5) Mabrouka and eight accessions 6, 7, 8, 9, 10, 11, 12 and 13 of mango with SDS-PAGE based on Jaccard's coefficients.

Genotype	13	12	11	10	9	8	7	6	5	4	3	2	1
13	1.000												
12	1.000	1.000											
11	1.000	1.000	1.000										
10	1.000	1.000	1.000	1.000									
9	0.970	0.970	0.970	0.970	1.000								
8	0.941	0.941	0.941	0.941	0.970	1.000							
7	0.941	0.941	0.941	0.941	0.970	1.000	1.000						
6	1.000	1.000	1.000	1.000	0.970	0.941	0.941	1.000					
5	0.914	0.914	0.914	0.914	0.941	0.971	0.971	0.914	1.000				
4	0.971	0.971	0.971	0.971	0.941	0.914	0.914	0.971	0.943	1.000			
3	0.971	0.971	0.971	0.971	0.941	0.914	0.914	0.971	0.943	1.000	1.000		
2	0.971	0.971	0.971	0.971	0.941	0.914	0.914	0.971	0.943	1.000	1.000	1.000	
1	0.971	0.971	0.971	0.971	0.941	0.914	0.914	0.971	0.943	1.000	1.000	1.000	1.000

the environment.

Conflict of Interests

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

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Full Length Research Paper

Segregation of vegetative and reproductive traits associated with tuber yield and quality in water yam (*Dioscorea alata* L.)

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Water yam (*Dioscorea alata* L.) is an important source of food for millions of people in Africa, Asia, South America, Caribbean and the South Pacific islands. Genetic mapping populations of this species have been produced as part of efforts to raise the efficiency of breeding through development and use of molecular markers. In this study, an F_1 mapping population (AM1) of *D. alata*, exhibited segregation for both tuber yield- and quality- related traits when evaluated in the field for 12 agronomic characters: days to shoot emergence, number of primary vines per plant, days to flowering, flower sex, flowering intensity, days to tuber initiation, number of tubers per plant, tuber yield per plant, tuber shape, bulbil formation, tuber browning and reaction to anthracnose (*Colletotrichum gloeosporioides* Penz) infection. The number of tubers/plant and the number of primary vines/plant were moderately positively correlated, while most of the other quantitative traits were weakly correlated with each other. There were more males (49%) than females (19.9%) identified in the population, however more than a quarter of the population (31%) did not flower and their sexes could not be determined. 20% of the progeny was resistant or tolerant to field infection by anthracnose. Population AM1 will be a valuable resource as a mapping population for genetic analysis and molecular marker development for tuber quality and several other agronomic traits in *D. alata*.

Key words: Flowering intensity, marker assisted breeding, mapping population, phenotypic variation and trait segregation, tuber yield and quality, water yam.

INTRODUCTION

Yam (genus *Dioscorea* in family Dioscoreaceae) is a polyploid and clonally-propagated crop that is cultivated for its starchy tubers. It is an important source of food and income for over 300 million people in Africa, Asia, South America, Caribbean and the South Pacific islands (Degras, 1983). It also has considerable socio-cultural significance, especially in West Africa and the South Pacific islands, where it is central to important annual ceremonies. Although there are more than 600 species of yam, only ten are generally cultivated for food including:

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Table 1. List of qualitatively scored traits assessed by using subjective scale during vegetative and reproduction growth and after harvest of *D. alata* mapping population AM1 and the two parents during 2010/2011 yam growing season at IITA, Ibadan, Nigeria. The assessment was performed in accordance with the IPGRI/IITA descriptors for yam.

Trait	Score code-descriptor state
Flower sex	Male = 1; Female = 2; Monoecious = 3; None = 4
Flowering Intensity	Very high flowering = 1; Moderate = 2; Scattered / Few = 3; None = 4
Tuber shape	Round = 1; Oval = 2; Oval-oblong = 3; Cylindrical = 4; Flattened = 5
Bulbil formation	High (>10 bulbils) = 1; Moderate = (6-10) = 2; Low (1-5) = 3; None = 4
Tuber oxidation	No oxidation = 0; Fast oxidation = 1; Slow oxidation = 2
Reaction to Anthracnose disease	Healthy (<2% or no symptom) = 1; Slightly infected (2-25% symptom) =2; Highly infected (>25% symptom) = 3

Dioscorea alata L. (water yam, greater yam), *D. rotundata* Poir. (white yam, white guinea yam) and D*. cayenensis* Lam. (yellow yam, yellow guinea yam) (Lebot, 2009).

D. alata was introduced to Africa from Asia during the 16th century (Hahn, 1995) and is the most widely distributed *Dioscorea* species in the tropics. It features high yield potential, ease of propagation (through production of bulbils and reliability of sprouting), early growth vigour for weed suppression and long storability of tubers. Although these characteristics are valuable for economic production, the species has major limitations in the field; including high susceptibility of most varieties to the devastating foliar disease, anthracnose caused by *Colletotrichum gloeosporioides* Penz, tendency towards poor or non-flowering, lack of synchrony in flowering of male and female genotypes and variation in flowering intensity with season and location (Hamadina et al., 2009). Tuber browning, caused by an enzyme catalysed oxidative reaction whereby a cut surface turns brown on exposure to air, is a problem in *D. alata* and other yam species (Martin and Ruberte, 1976), affecting their eating quality. Analysis of tuber yield-related traits in eight accessions of *D. alata,* comprising five breeding lines and three landrace varieties identified shoot dry weight and time of shoot emergence as traits related to fresh tuber yield (Sartie et al., 2011). Shoot dry weight had the strongest positive effect, while time of shoot emergence had a negative effect.

As genetic improvement of yam through conventional breeding alone is difficult and slow, efforts are being made to introduce marker-assisted selection to improve the efficiency of yam breeding programmes. Molecular markers, including simple sequence repeats (SSRs) and single nucleotide polymorphism (SNPs) are being deve-loped (Bhattacharjee et al., 2013; Tamiru et al., 2013) for the purpose of screening genetic mapping populations segregating for key traits of interest and identifying markers linked to those traits for use as aids in selection by breeders. As a previous F_1 mapping population deve-loped for *D. alata* (Mignouna et al., 2002) has been lost, new mapping populations have recently been developed from breeding germplasm at the International Institute of Tropical Agriculture (IITA) for marker discovery in *D. alata* and *D. rotundata* (Sartie and Asiedu, 2011).

The aim of this research was to evaluate a *D. alata* L. genetic mapping population for phenotypic variation in vegetative and reproductive traits associated with tuber yield, tuber quality and anthracnose disease resistance.

MATERIALS AND METHODS

Yam genotypes and experimental design

Seventy eight (78) out of 144 genotypes from the genetic mapping population AM1 (Sartie and Asiedu, 2011) and the two parents were assessed in the field from planting to tuber harvest during the 2010/2011 yam growing season at IITA, Ibadan, Nigeria. Population AM1 is an F_1 full-sib population developed from crossing *Dioscorea alata* L. accessions TDa 01/00081 and TDa 87/01091, which differ in breeding traits of interest. For each genotype, 6 to 15 tuber setts weighing 600 g were prepared and buried in carbonized rice husks for sprouting. The sprouted setts were transplanted into mounds (about 30 cm high) in the field at a spacing of 0.5 m × 1 m in α-lattice design with two replications of three plants per genotype in April 2010. The remaining setts and the 66 genotypes that had less than 5 setts were planted at the borders of the experiment for tuber multiplication. Harvest was in February 2011.

Trait phenotyping

Twelve agronomic traits were assessed: days to shoot emergence, number of primary vines per plant, days to tuber initiation, days to flowering, flowering intensity, flowering sex, number of tubers per plant, tuber yield per plant, tuber shape, tuber oxidation, bulbil formation, and reaction to anthracnose infection. Of these, six traits (Table 1): flowering intensity, flowering sex, bulbil formation, tuber shape, tuber oxidation and reaction to anthracnose infection were scored using qualitative (subjective) scales, following the internationally agreed descriptor list for yam (IPGRI/IITA, 1997). Tuber oxidation was scored by visual observation of tuber parenchyma 2 min after cutting the fresh tuber, and was performed at the time of preparation of setts for planting each genotype. Anthracnose resistance phenotyping involved scoring for both the minimum and maximum symptoms observed on a naturally infected plant on the field. Days to shoot emergence, number of primary vines per plant, days to tuber initiation, days to flowering, number of tubers per plant, and tuber yield per plant were scored quantitatively as follows:

Days to shoot emergence

This was recorded as the number of days between the planting of a

Table 2. Phenotype of parents and progeny of 6 qualitatively scored traits assessed in mapping population AM1 in 2010/2011 yam growing season. Phenotypic proportion was calculated as percentage of setts from progeny and two parental lines in each qualitative trait category.

Qualitatively scored trait	Parental phenotype		Progeny phenotype
	TDa 01/00081 (♀)	TDa 87/01091 (♂)	Phenotype
Flowering sex	Female (50%), None (50%)	Male (66.7%), None (31.0%)	Male (49.0%), None (31.0%), Female (19.9%)
Flowering intensity	Very high (60.0%), None (40%)	Scattered/few (66.7%), None (33.3%)	None (38.5%), Moderate (23.1%), Very high (21.6%), Scattered/few (16.9%)
Tuber shape	Round (50%), Oval (50%)	Cylindrical (66.7%), Oval oblong (33.3%)	Oval oblong (39.8%), Cylindrical (25.4%), Oval (24.9%), Round (6.24%), Flattened (3.7%)
Bulbil formation	None (100%)	None (50%), Low (33.3%), High (16.7%)	None (50.1%), Low (29.1%), High (11.6%), Moderate 99.2%)
Tuber oxidation	No oxidation (100%)	Slow/slight oxidation (100%)	No oxidation (60.7%), Slow/slight oxidation (34.8%), Fast oxidation (4.5%)
Anthracnose min	Slightly Infected (66.7%), healthy (33.3%)	Healthy (100%)	Healthy (90.8%), Slightly Infected (9.2%)
Anthracnose max	Slightly Infected (100%)	Slightly Infected (100%)	Slightly Infected (78.4%), Healthy (19.9%), Highly Infected (1.7%)

sprouted tuber sett and the emergence of a shoot above the ground.

Number of primary vines per plant

This was determined by counting the number of primary vines (stems) produced per plant at 20 days after emergence.

Days to tuber initiation

The bases of the plants were exposed six weeks after planting and inspected for the presence of new tubers every other day until tubers were observed for all the plants. The soil was carefully opened and closed to avoid root damage. The six weeks starting time was chosen based on a previous study (Sartie et al., 2011) in a population of breeding lines and landrace varieties of *D. alata* that showed that tubers were not initiated before 55 days after planting in two consecutive years.

Number of tubers per plant

This was determined by counting the harvested tubers per plant.

Days to flowering

These were counted as the numbers of days between shoot emergence and flowering.

Tuber yield per plant

The weight (kg) of tubers per plant was measured immediately after harvest.

Ploidy levels for all the progeny and parents were determined using a flow cytometer (Obidiegwu et al., 2009).

Statistical methods

For the qualitatively scored traits, the number of setts with results in each of the categories was tallied and converted to a percentage of the total number of setts with values (missing values were not used in the total).

For the quantitatively scored traits, variance components (Robinson, 1987) were calculated using a linear mixed model with random terms for clone, and replication within each clone. The variation among the setts within a plot was used as the residual. Narrow-sense heritability, h^2 was calculated from the variance components in two ways: 1) h^2 = clone / (clone + residual); 2) h^2 = clone / (clone + rep + residual).

Comparisons between the two parental lines (TDa 01/00081 and TDa 87/01091) were performed using a linear mixed model (Patterson and Thompson, 1971) with a fixed term for clone and a random term for replicate, for each of the quantitatively scored variables.

To calculate summary statistics for the progeny, the results (of the three setts) were first averaged for each plot, and then summary statistics (mean, minimum and maximum) were calculated using the averages. The averages were tested for normality using the Anderson-Darling test, and Spearman rank correlations were calculated between the variables.

RESULTS

Qualitatively scored traits

Considerable variation was observed amongst the progeny for all the traits (Table 2). For most of the traits (flowering intensity, tuber shape, bulbil formation, tuber browning and reaction to anthracnose infection), a proportion of the progeny population exhibited a phenotype that was absent in either parents (female TDa 01/00081 and male TDa 87/01091).

Among the plants that flowered, 49% were males and 19.9% females, however more than a quarter of the population (31%) did not flower (Table 2 and Figure 1a). About 45% of progeny population produced flowers moderately, or profusely (Table 2, Figure 1b). Tuber

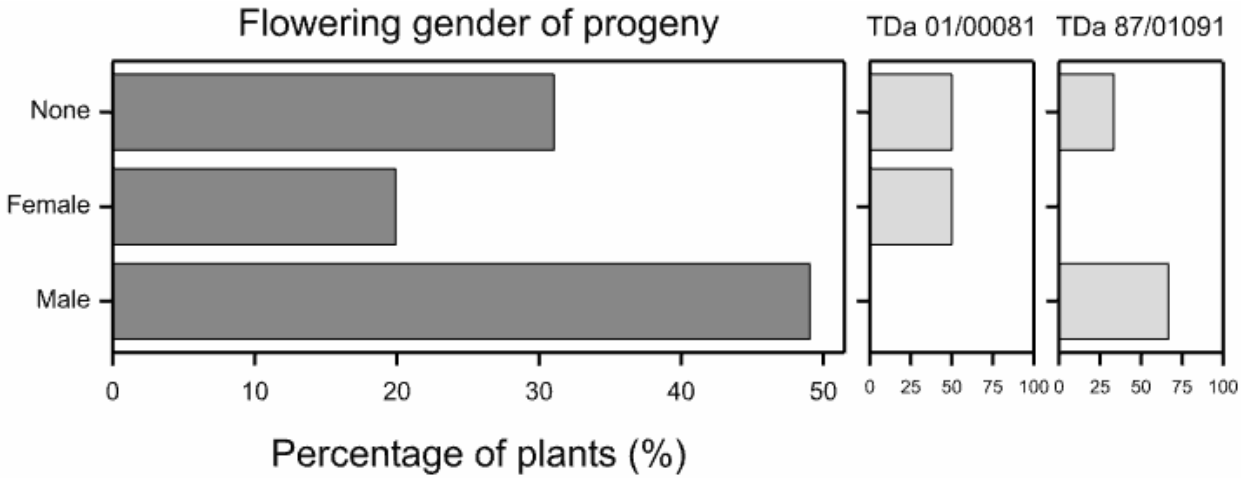

Figure 1a. Flowering and sex of plants that flowered.

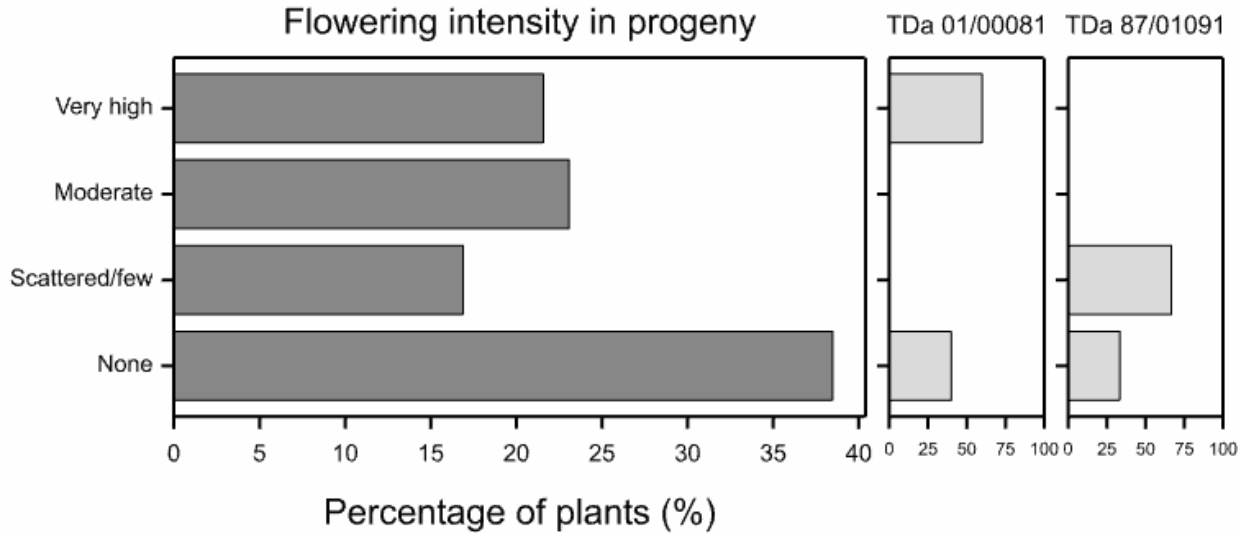

Figure 1b. Percentage of plants from progeny and two parental lines in each flowering intensity category.

shape (Table 2 and Figure 1c) varied the most in the population, with five categories observed. Nearly half of the population did not produce bulbils (Table 2 and Figure 1d), but a reasonable proportion of the progeny (11.6%) produced more than 10 bulbils/plant. Tuber browning is associated with poor eating quality in yam, and the trait segregated in the progeny at three levels with 61% of the population producing tubers that did not turn brown upon exposure of cut surface to the atmosphere (Table 2 and Figure 1e). Assessment based on maximum infection scores shows that only 20% of the genotypes were resistant, or tolerant to anthracnose disease (Table 2 and Figure 1f), as they were either healthy, or had less than 2% symptoms of the disease (Table 1). A small proportion of the progeny (6.6%) produced tubers with short dormancy that sprouted before they were harvested. The parents differed in flowering sex, flowering intensity, tuber shape, bulbil formation and tuber oxidation, (Table 2 and Figure 1), but were similar in levels of anthracnose infection (Figure 1f).

Quantitatively scored traits

Summary statistics for the progeny means for the parents and the results of statistical comparisons between the parents are shown in Table 3. The parents differ significantly ($p < 0.05$) in tubers/plant and primary vines/plant. Histograms of the results for the progeny are shown in Figures 2a to 2f. Although the results of Anderson-Darling tests for normality of the progeny results show that all but one variable, tuber yield/plant, differ significantly from being normally distributed (Table 3), normal probability Q-Q plots of the progeny results (Data not shown) suggest

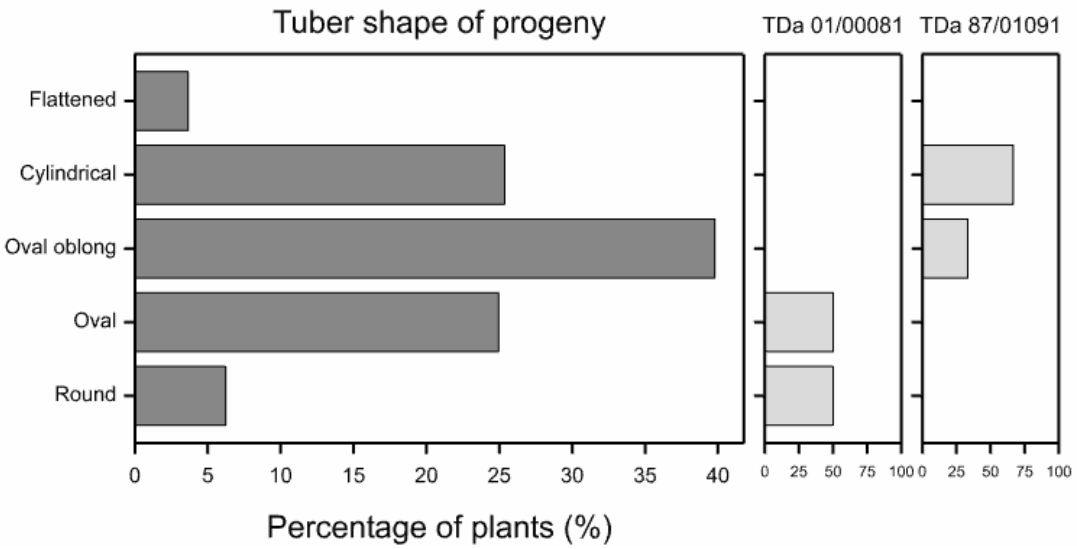

Figure 1c. Percentage of plants from progeny and two parental lines in each tuber shape category.

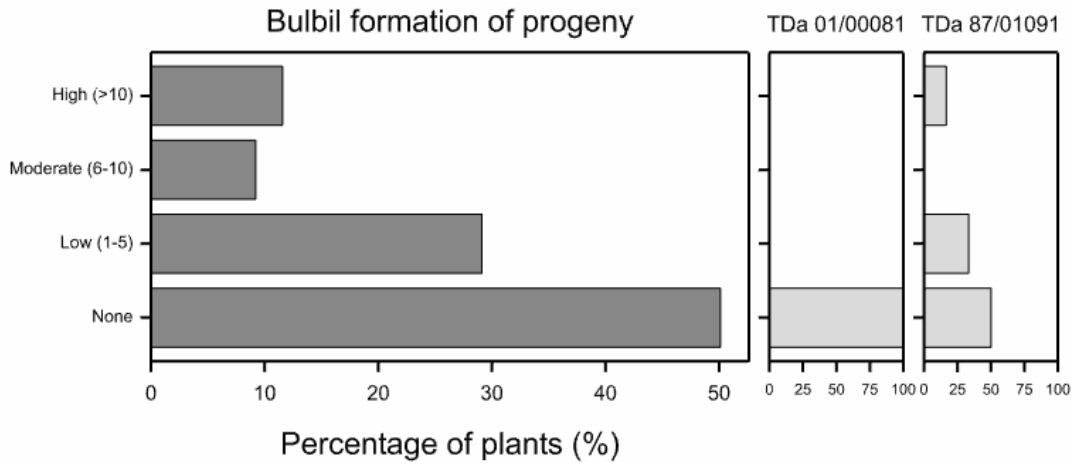

Figure 1d. Percentage of plants from progeny and two parental lines in each bulbil formation category.

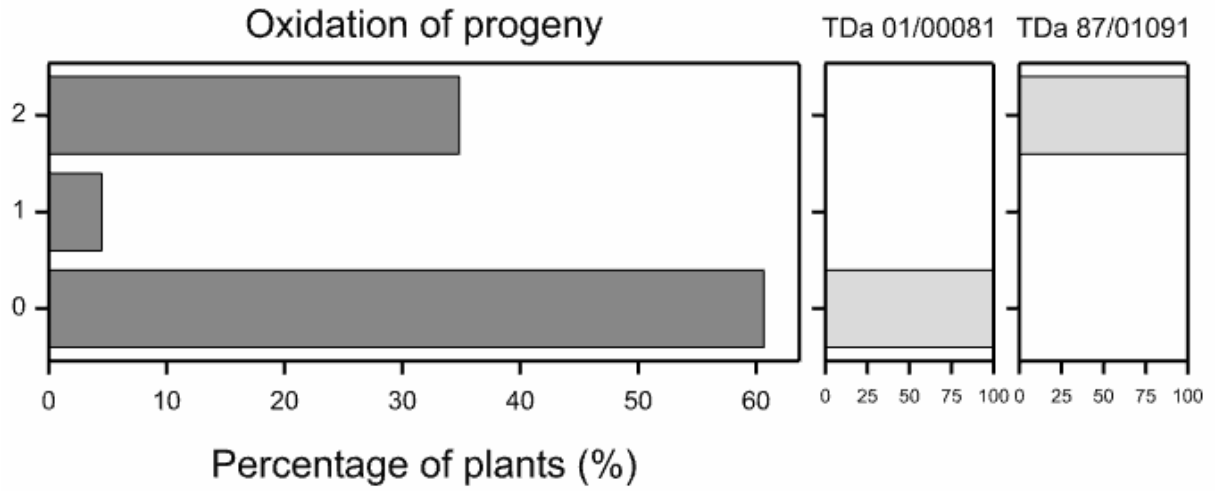

Figure 1. Percentage of plants from progeny and two parental lines in each oxidation category.

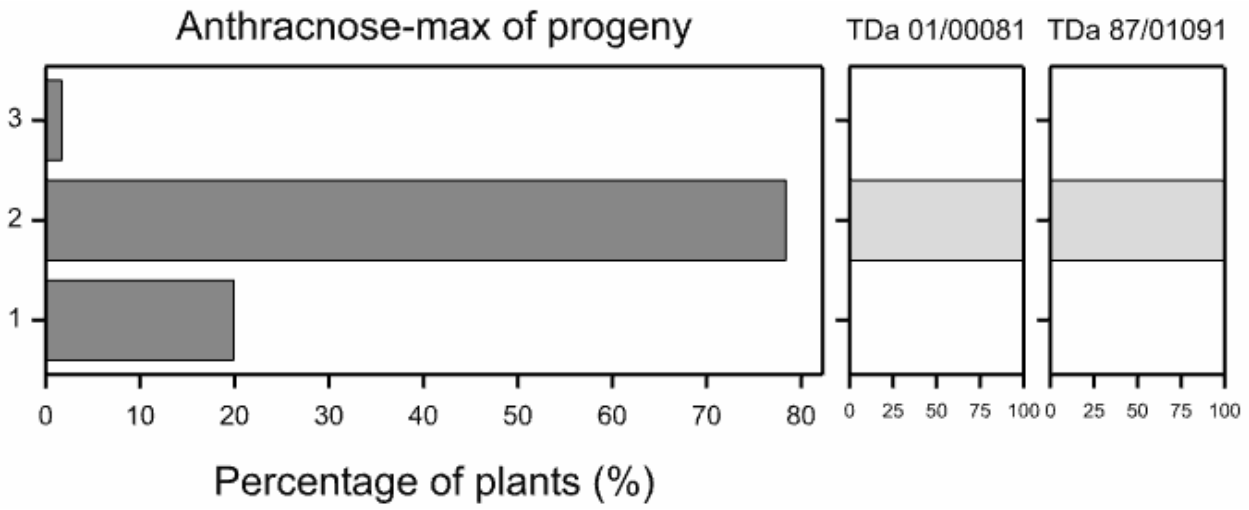

Figure 2. Percentage of plants from progeny and two parental lines in each anthracnose-maximum category.

Table 3. Summary statistics (mean, minimum, maximum) and normality for quantitative traits assessed in progeny and parents of mapping population AM1 during 2010/2011 yam growing season. P^1 is for comparison of parent means using a linear mixed model. Anderson-Darling *A* tests for normality of quantitative traits measured from progeny. Variables with P-values <0.05 are considered significantly different from normally-distributed.

Quantitatively scored trait	Progeny results			TDa 87/01091	TDa 01/00081	P^1	Normality	
	Mean	Minimum	Maximum	Mean	Mean		*A*	*P*
Days to shoot emergence	19.3	13.0	30.7	22.0	24.3	0.59	0.91	<0.03
Primary vines/plant	5.3	1.3	20.3	3.0	4.2	0.058	8.46	<0.01
Tubers/plant	2.6	1.0	6.0	2.0	2.7	0.025	3.46	<0.01
Days to tuber initiation	82.8	75.0	137.0	80.0	84.5	0.14	6.76	<0.01
Days to flowering	133.9	90.3	154.0	139.5	128.7	n/a	1.46	<0.01
Tuber yield/plant (Kg)	3.6	0.3	7.8	3.3	3.2	0.89	0.49	>0.15

n/a = P-value is not available because one parent had results from only one rep.

Table 4. Spearman rank correlations between quantitatively scored traits assessed in AM1 yam mapping population during 2010/2011 yam growing season. Values in parentheses are p-values.

Quantitatively scored variable	Days to shoot emergence	Number of primary vines/plant	Number of tubers/stand	Days to tuber initiation	Days to flowering after emergence
No. primary vines/plant	-0.27 (0.003)	-			
No. tubers/stand	-0.29 (0.001)	0.54 (<0.001)	-		
Days to tuber initiation	-0.01 (0.95)	-0.08 (0.39)	0.15 (0.10)	-	
Days to flowering after emergence	-0.36 (<0.001)	0.21 (0.021)	0.22 (0.016)	-0.09 (0.31)	-
Tuber yield/stand	-0.10 (0.29)	0.02 (0.79)	0.01 (0.95)	-0.28 (0.002)	0.22 (0.015)

that days to shoot emergence and days to flowering after emergence are not practically different from being normally distributed.

Most of the quantitatively scored traits were only weakly correlated with each other (Table). The number of tubers/plant and the number of primary vines/plant were moderately positively correlated (r = 0.54). Tuber yield correlated negatively (p = 0.002) with days to tuber initiation but positively (p = 0.015) with days to flowering.

Days to shoot emergence correlated negatively (p < 0.001) with days to flowering and tuber number/stand replicates of the same clone, and the “residual” component is the variance among the setts within a plot.

When calculating narrow-sense heritability, the “rep”

Table 5. Variance components and narrow-sense heritability for quantitative traits assessed in mapping population AM1 in 2010/2011 yam growing season. The narrow-sense heritability is calculated using the variance components in two ways: h^2 = clone / (clone + residual); and h^2 = clone / (clone + rep + residual).

Quantitative Trait	Variance component			Narrow-sense heritability	
	Clone	Rep	Residual	h^2	h^2
Days to shoot emergence	0.200	0.00001	30.141	0.007	0.007
No. primary vines/plant	8.567	0.437	4.907	0.636	0.616
No. tubers/stand	0.697	0.000	1.655	0.296	0.296
Days to tuber initiation	23.167	23.167	12.494	0.650	0.394
Days to flowering after emergence	35.654	30.599	74.289	0.324	0.254
Tuber yield/stand	1.316	0.199	1.617	0.449	0.420

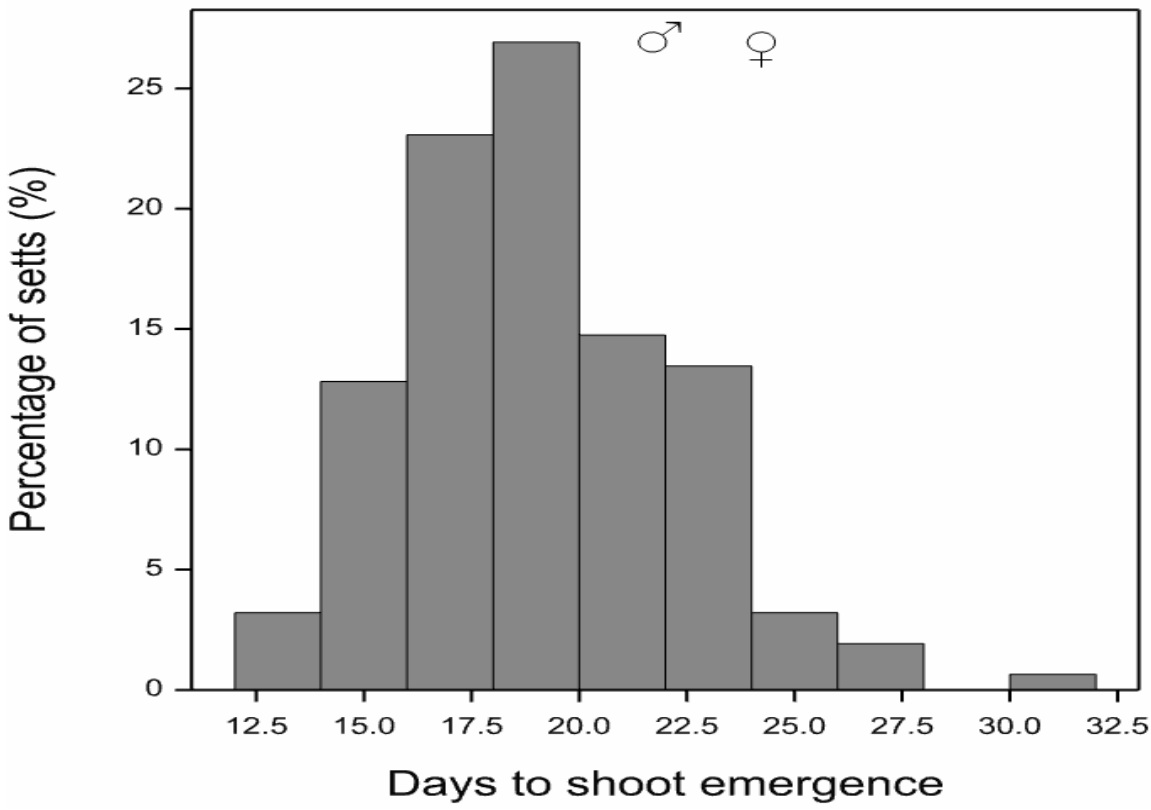

Figure 2a. Histogram of days to shoot emergence results from all progeny and the two parents (♀ = female parent and ♂ = male parent).

variance can either be included in the total variance (the denominator of the fraction) or not. Heritability was calculated in both ways (**Error! Reference source not found.**5), and was moderately high for most of the traits, but very low for days to shoot emergence.

Ploidy analysis

Ploidy analysis results show that two of the progeny had ploidy levels of 2n = 80 (8x), while the rest, including their parents, had 2n = 40 (4x). The two 8x genotypes, both males, did not express a phenotype significantly different from the other genotypes.

DISCUSSION

Considerable variation was observed among the progeny for the 12 traits that were assessed. Earlier results have shown the segregation of parental alleles in population AM1 when DNA of six selected progenies and the two

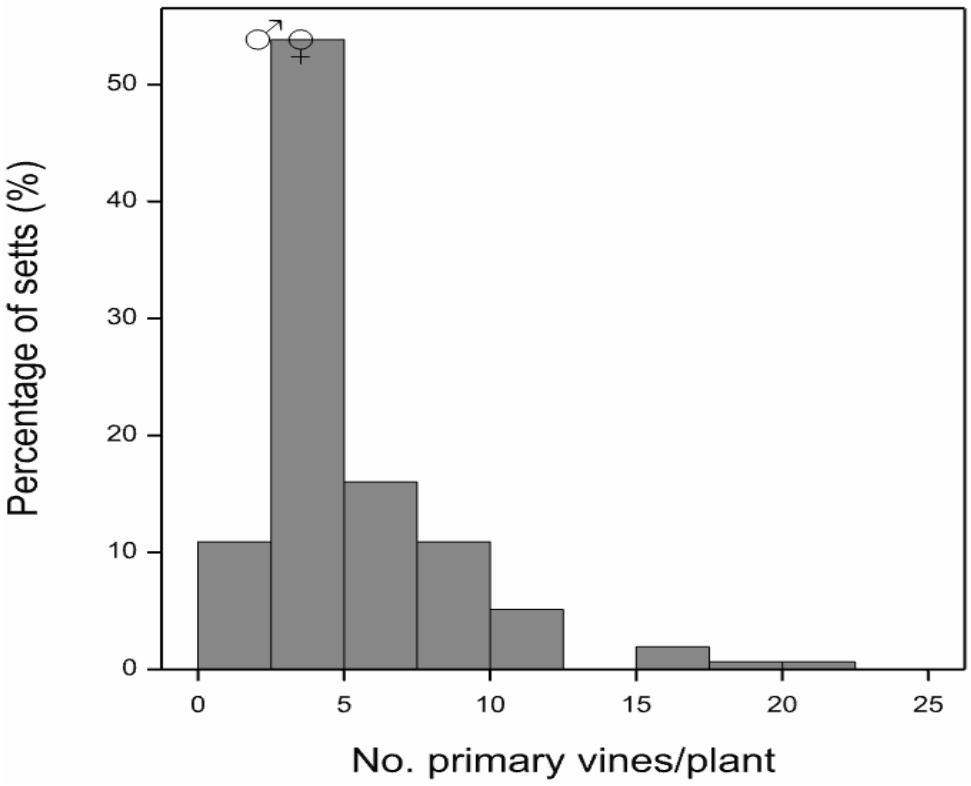

Figure 2b. Histogram of results from all progeny and the two parents for number of primary vines per plant (♀ = female parent and ♂ = male parent).

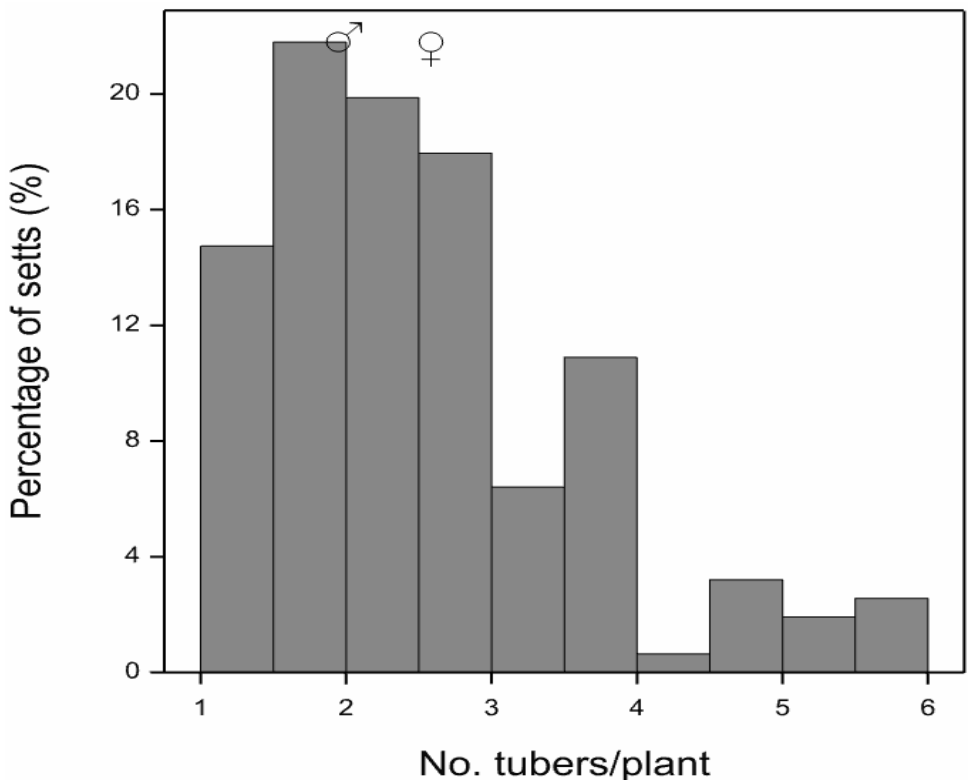

Figure 2c. Histogram of results from all progeny and the two parents for number of tubers per stand (♀ = female parent and ♂ = male parent).

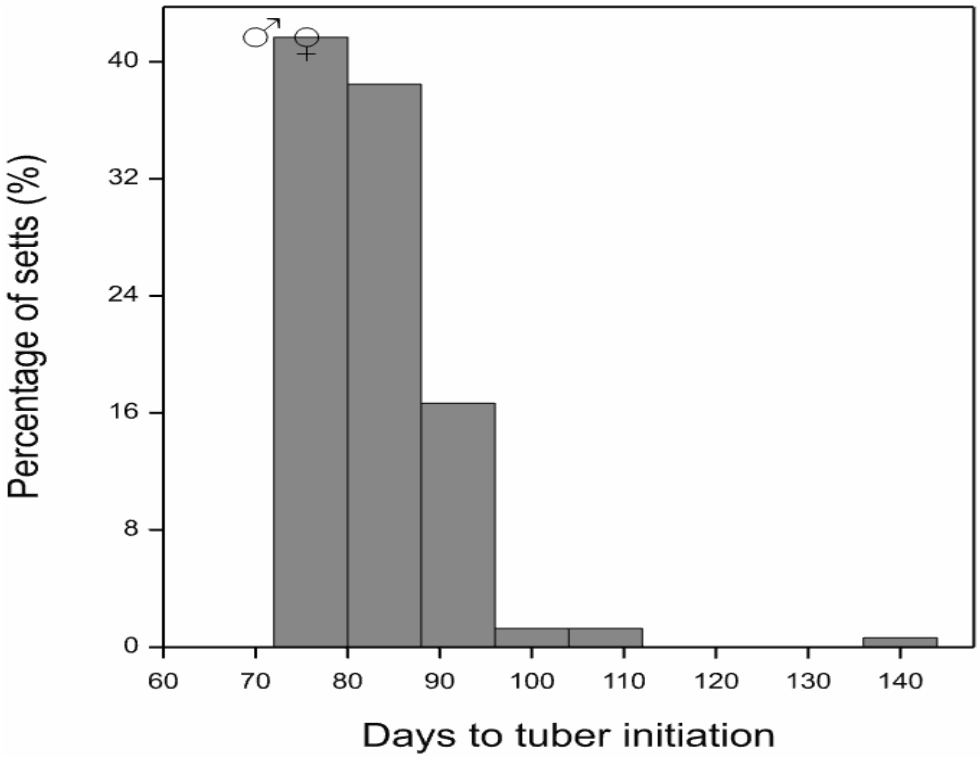

Figure 2d. Histogram of results from all progeny and the two parents for days to tuber initiation (♀ = female parent and ♂ = male parent).

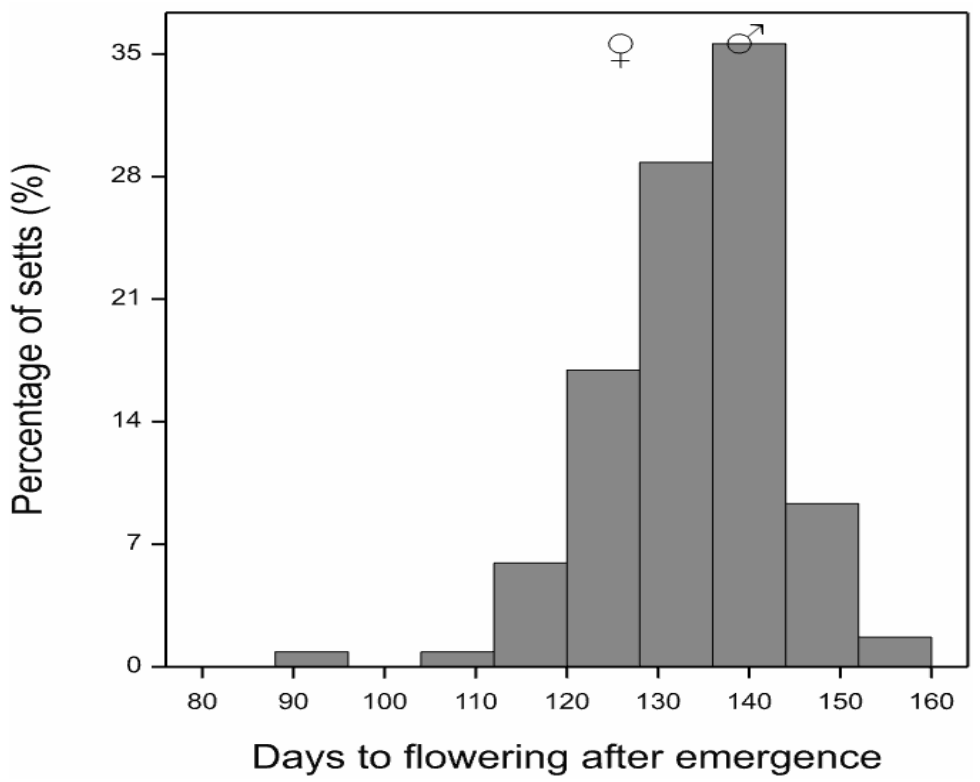

Figure 2e. Histogram of results from all progeny and the two parents for number of days to flowering after emergence (♀ = female parent and ♂ = male parent).

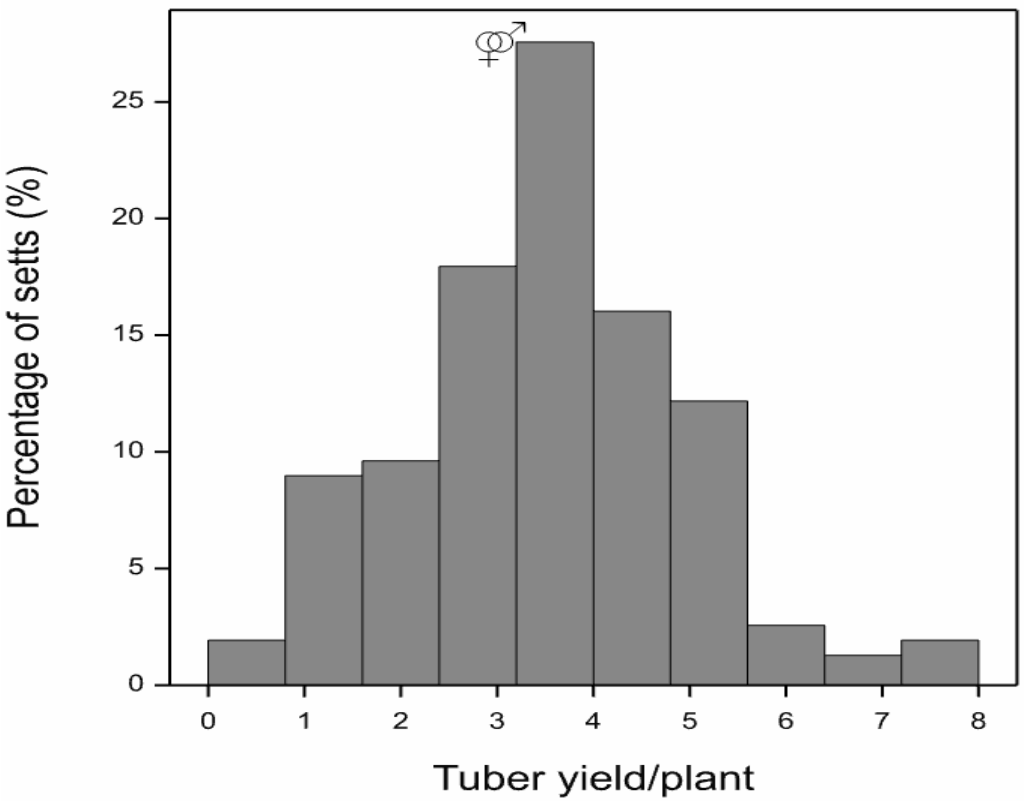

Figure 2f. Histogram of results from all progeny and the two parents for tuber yield per stand (♀ = female parent and ♂ = male parent).

parents were analysed with SSR markers (Sartie and Asiedu, 2011). The findings taken together indicate that population AM1 should be suitable for genetic mapping and development of markers linked to these traits in *D. alata*. Transgressive segregation (the production of extreme phenotypes in the progeny compared to the parental lines) that was observed for some of the traits (flowering intensity, tuber shape, bulbil formation, tuber oxidation and reaction to anthracnose disease), represents a potential source of novel genetic variation in hybrids that may be significant for the genetic improvement in these traits (de Vicente and Tanksley, 1993). Correlation analysis shows a weak association among most of the traits, which suggest that they are largely independent of one another, except for tuber yield-related traits that were shown to be moderately correlated. As the current investigation was conducted for only one year in a partial population of 78 out of 144 progeny of mapping population AM1, it is highly desirable that these preliminary results are validated in the whole population over two years, before genetic mapping proceeds.

Tuber yield- related traits in *D. alata* had been previously identified as shoot dry weight and time of shoot emergence, with tuber yield correlating positively with shoot dry weight, but negatively with time of shoot emergence (Sartie et al., 2011). Days to flowering was not assessed in the previous study, while shoot dry weight was not investigated in the current study. The previous study was undertaken in a smaller population of eight *D. alata* accessions of cultivars. Results based on findings of the current and previous (Sartie et al., 2011) studies in two populations of different genetic backgrounds, indicate that tuber yield-related traits in *D. alata* include: number of primary vines/plant; days to shoot emergence; shoot dry weight; days to tuber initiation; and days to flowering. Days to tuber initiation and shoot emergence correlated negatively with tuber fresh weight in both studies, indicating that delayed shoot emergence or tuber initiation may result in low tuber yield. Moreover, the low heritability of days to shoot emergence suggests that this trait may be largely influenced by the environment.

Extent of flowering affects the ease and success of developing breeding and mapping populations in yam. Some genotypes do not flower, or they flower only sparingly. Extent of flowering may also be affected by the environment, such as season and location (Hamadina et al., 2009). In the current study, more than a quarter of the population (31%) did not flower (Table 2 and Figure 1a), and their sex could not be determined. This poses a challenge for developing hybrid populations in *D. alata*. Assessment of population AM1 for flowering at multi-locations and across seasons or years will be useful for

the identification of genotypes that flower every year, or only seasonally.

Tuber shape is an important trait for marketing and exporting of yam tubers. Variable tuber shapes including round, oval, oval oblong, cylindrical and flattened segregated in the study population (Table 2 and Figure 1c), which indicates that population AM1 can be used for developing cultivars of specific shapes. Tuber shape, however, can be manipulated artificially, as some farmers manage the process of changing the form by adjusting the type of the mounds, while others have introduced flat stones into the mounds to produce short and fat tubers (Zannou et al., 2006).

The availability of seed tubers for planting is a major limitation in yam production, due to low seed multiplication factor of the crop, on one hand, and that the tuber is the edible part used as food, on the other hand. Bulbils are aerial tubers that can be used as seeds in crop production. About 40% of genotypes in the study population produced bulbils (Table 2 and Figure 1d), which would serve as complementary source of seed tubers. The segregation of bulbil formation in this population therefore confirms the suitability of population AM1 for genetic analysis and marker development for bulbil formation in *D. alata*. Phenotypic expression for bulbil production suggests that the trait may be influenced by environment, as shown in the parent that produced bulbils.

Tuber browning (discoloration of yam when a cut surface turns brown on exposure to air), which is associated with poor eating quality is a problem in *D. alata* (Martin and Ruberte, 1976). In this study, 61% of the genotypes in mapping population AM1 produced tubers that were non-browning, while the others were associated with fast or slow browning (Table 2 and Figure 1e). Population AM1 is therefore a potential gene pool for breeding for non-browning combined with other desirable traits in *D. alata* and it would also be suitable for genetic analysis and marker development for tuber browning in *D. alata*.

Anthracnose is a major disease that affects the production of *D. alata*, with the symptoms of this disease visible on the leaves of infected plants. Evaluating a yam plant for disease incidence on the field requires scoring for both minimum and maximum infection, due to the often non-uniform distribution of disease in field plots.

Ideally highly resistant clones are expected to have relatively low to moderate scores even for the maximum infection reading. Trying to give an average reading by just looking at the field will not capture the potential maximum severity the clone could suffer when under intense pressure. This indicates that phenotyping anthracnose disease based on natural infection on the field is difficult and sometimes misleading. Phenotyping of this disease could be more reliable following inoculation of plants under controlled conditions as reported previously for *D. alata* (Mignouna et al., 2001) and for other diseases in other crops, for example *Pseudomonas syringae* pv. *actinidae* (*Psa*) in Kiwifruit (*Actinidia* sp.) (Gardiner et al., 2013) and Blast (*Magnaporthe oryzae* (Hebert) Barr.) and Bacterial leaf blight (*Xanthomonas oryzae* pv. oryzae (*Xoo*) of rice (*Oryza* sp.) (Pinta et al., 2013). Our result shows that some genotypes of the study population were resistant, or tolerant to anthracnose infection, whilst neither of their parents showed complete resistance (Table 2 and Figure 1f). This indicates that although population AM1 might not be best suited for mapping markers for resistance, it may be a beneficial gene pool to facilitate genetic improvement for anthracnose resistance in this species.

Long tuber dormancy (which may last over 4 months), coupled with a long growth and development period of about 7 months (Sartie et al., 2011), prevents the production of *D. alata* more than once or twice in a year, which hinders fast improvement and increased production of the crop. A small proportion (about 7%) of population AM1 had their tubers sprouted before they were harvested, which indicates the possibility of double cultivation of those genotypes with short dormancy, or using them as parents for developing cultivars for multiple production within a year, providing that the other growth factors are available. However, short tuber dormancy would mean short tuber shelf-life, which may reduce consumer preference.

Good cooking quality is a valuable characteristic in cultivar development in yam. Parents of mapping population AM1 differ on cooking quality (Sartie and Asiedu, 2011), with a possibility for the trait to segregate in the population. However, as cooking quality was not assessed in the current investigation, it would be necessary to evaluate population AM1 for this trait in future studies.

D. alata includes varieties with three ploidy levels of 2n = 40, 60 and 80 chromosomes, which have been previously classified as tetraploid, hexaploid and octaploid respectively, with x = 10 as basic chromosome number (Abraham and Nair, 1991; Gamiette et al., 1999; Malapa et al., 2005; Obidiegwu et al., 2009). However, genetic analysis with Amplified Fragment Length Polymorphic (AFLP) markers in F_1 hybrids of *D. alata* revealed that the markers segregated as in a diploid cross-pollinated population (Mignouna et al., 2002). A recent review on ploidy status of *D. alata* using cytogenetics as well as SSR markers (Arnau et al., 2009) has also established that plants with 2n = 40 chromosomes are diploids with the basic chromosome number of x = 20. In the current study, most (97%) of the progeny were 2n = 40 as were their parents, indicating that mapping population AM1 is a diploid population derived from diploid parents.

Conclusions

Mapping population AM1 is a valuable resource for QTL mapping and genetic marker development for marker-assisted breeding for tuber yield, tuber shape, bulbil formation, tuber browning and flowering intensity in water

yam (*D. alata)*. There were considerable phenotypic variations amongst the progeny for all the assessed traits, with some progeny showing extreme phenotypes that were not expressed in the parents. Ploidy analysis indicated that mapping population AM1 is a diploid population derived from diploid parents.

Conflict of Interests

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

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Full Length Research Paper

Can hydrogen peroxide and quercetin improve production of *Eucalyptus grandis* x *Eucalyptus urophylla*?

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Vegetative propagation is considered the best choice for the rapid multiplication of plant species, however, rooting may still present difficulties. Substances, such as auxins, phenolic compounds and hydrogen peroxide, are recognized as able to improve this process. The aim of the present work was to determine if hydrogen peroxide in combination with quercetin or indole butyric acid, can modify some characteristics related to rooting and development in cuttings of *Eucalyptus grandis* x *Eucalyptus urophylla*. Cuttings were periodically evaluated at 30, 60 and 90 days according to the following criteria: height, diameter and survival percentage. After planting (90 days), a destructive evaluation was performed to determine rooting percentage, average size and number of roots. Polyamines content and polyamine oxidase activity, as biochemical markers of plant development, were determined. No statistically significant differences in height, diameter, survival and rooting percentage, root length and number of roots per cuttings were found. Treatments induced a decrease in putrescine levels and polyamine oxidase activity in roots. For absence of positive responses, the use of these substances as a treatment to improve cutting production is economically unviable.

Key words: Phenolic compounds, indole butyric acid, vegetative propagation, cuttings.

INTRODUCTION

Vegetative propagation is considered an ideal choice for the rapid multiplication of species, which maintain desirable characteristics. However, rooting of tree species by cuttings may be hampered by several factors, such as the cutting position, the age of the donor plant and possible treatments with indole butyric acid (IBA) (Hartmann et al., 2002).

Hormones and plant growth regulators, such as synthetic auxins (IBA) play complex roles on rooting and can have direct (cell growth or division) and indirect (interacting with other molecules or plant hormones) effects (Corrêa and Fett-Neto, 2004).

Some substances, such as phenolic compounds and

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hydrogen peroxide has been identified as important regulators of auxin production in plants. Some phenolic groups, such as p-diphenols, o-diphenols, polyphenols, coumarins and flavonoids, namely quercetin, are described as inhibitors of indoleacedic acid (IAA) oxidation (Banduski et al., 1995) by inhibition of IAA oxidase (Pandey and Pathak, 1981).

The accumulation of H_2O_2 during root formation and its beneficial effects, at low concentrations, has been pointed out in several studies (Dunand et al., 2007; Li et al., 2009). Improvements in rooting were described when this substance was associated with auxins (Sebastiani and Tognetti, 2004; Franklin and Dias, 2011).

PAs play critical roles in a variety of physiological and developmental processes, such as regulation of cell proliferation, somatic embryogenesis, differentiation and morphogenesis (Kusano et al., 2008). Relationships between polyamines and different aspects of root development, such as increases in meristematic activity, elongation, lateral and adventitious roots formation have been discussed in several studies (Couée et al., 2004). The addition of PAs significantly improved root formation and/or growth in sweet orange, depending on their concentration, whereas the presence of inhibitor of PAs biosynthesis, such as α-difluoromethylornithine (DFMO), inhibited these processes (Mendes et al., 2011). Agmatine (Agm), spermidine (Spd) and spermine (Spm) levels were positively related to root development, while the content of putrescine (Put) has neutral or negative effect (Su et al., 2006). Moreover, PAs are known to play a role against oxidative damage. This antioxidant effect is probably due to the combination of their free radical scavenging properties inhibiting lipid peroxidation reactions catalyzed by metals, and the production of hydrogen peroxide by the action of amine oxidases (Groppa and Benavides, 2008). Polyamine oxidases (PAOs) (EC 1.5.3.11) are responsible for catalyzing the oxidation of polyamines, such as spermine (Spm) and spermidine (Spd), and/or of their acetylated derivatives (Angelini et al., 2010). These enzymes present a direct relationship with auxin content in plants. The application of exogenous auxin can induce an increased expression of PAOs during growth of corn mesocotyls (Cona et al., 2003). In a previous study, it was demonstrated that the enzymes polyamine oxidase and diamine oxidase (DAO/PAO) are inversely related to endogenous polyamines levels in the rooting induction phase. Hydrogen peroxide levels were positively related to DAO and PAO activity, as one of the final products of polyamines oxidation (Nag et al., 2001).

The great interest in the improvement of the quality of *Eucalyptus* seedlings and the good results obtained in previous studies on rooting, led us to the present experimental work aimed at evaluating the levels of polyamines on the basis of the effect of hydrogen peroxide responses, the flavonoid quercetin, and indole butyric acid during the rooting and development of cuttings of *E. grandis x E. urophylla.*

MATERIALS AND METHODS

Cuttings of *E. grandis x E. urophylla,* clone (CL1), from Duratex S.A. (Sao Paulo, Brazil) were used for the experiments, which were carried out at the Central Nursery farm of Duratex corporation, located in the municipality of Lençóis Paulista, Sao Paulo, Brazil, at 22º35'55" South latitude and 48º48'01" West longitude, 560 m altitude, in the Midwest region of the Sao Paulo state. The region present an Aw (tropical) climate, according to the Köppen classification, with 1133 mm annual average rainfall, 23.3°C mean annual temperature, with 29.1 and 17.6°C as maximum and minimum values, respectively.

Cuttings collection

The collection of cuttings, 3-6 cm long, was accomplished at Duratex S.A.in cement channels, 90 cm wide. Cuttings were picked up from 20 cm strains. Scissors were sterilized with 70% alcohol to prevent contamination by pathogens and a cooler, in which the cuttings were placed and used for data collection. The cuttings were sprayed with calcium plus boron (Ca + B), at 15 ± 5°C, every 10 min, to maintain the redox conditions of swelling solution. Cuttings, obtained from *E. urophylla x grandis* clones (CL1), were standardized in sizes from 3 to 6 cm long and 1.5 to 2.5 mm in diameter. Stakes were free of pathogens and presented one to three leaf pairs. Half of leaf area was removed to decrease excess sweating and water loss. The period spent under cutting collecting was always lower than 30 min to minimize cutting loss by oxidation.

Tillage treatments

Treatment solutions, in which cuttings were dipped, were freshly prepared. In this study three experiments were performed. In the first experiment, due to the low solubility in water, 98% quercetin (Sigma, Brazil) and indole butyric acid (IBA) 98% (Sigma, Brazil) were dissolved in acetone, and then mixed with $CaSO_4$ in different combinations for following treatments: T1– control; T2 – quercetin (0.25 g kg^{-1}); T3 - quercetin (0.5 g kg^{-1}); T4 – quercetin (1.0 g kg^{-1}); T5 – IBA (1.0 g kg^{-1}); T6 - IBA (1.0 g kg^{-1}) + quercetin (0.25 g kg^{-1}); T7 - IBA (1.0 g kg^{-1}) + quercetin (0.5 g kg^{-1}); T8 - IBA (1.0 g kg^{-1}) + quercetin (1.0 g kg^{-1}).

For the peroxide second experiment, 30% hydrogen peroxide (Merck, Brazil) (v/v) and IBA, mixed with $CaSO_4$ in the following combinations, were used: T1 – control; T2 - hydrogen peroxide (1.75%); T3 - hydrogen (3.5%); T4 - hydrogen peroxide (7.0%); T5 - IBA (1.0 g kg^{-1}); T6 - IBA (1.0 g kg^{-1}) + hydrogen peroxide (1.75%); T7 - IBA (1.0 g kg^{-1}) + hydrogen peroxide (3.5%); T8 - IBA (1.0 g kg^{-1}) + hydrogen peroxide (7.0%).

The third experiment was carried out by combining the most appropriate treatment with hydrogen peroxide and quercetin, from previous experiments, and associated with IBA, according to the following combinations: T1 - control; T2 - quercetin (0.5 g kg^{-1}) + hydrogen peroxide (3.5 %); T3 - IBA (1.0 g kg^{-1}); T4 - quercetin (0.5 g kg^{-1}) + hydrogen peroxide (3.5%) + IBA (1.0 g kg^{-1}).

Experimental design

For the implementation of the first two experiments, a completely randomized design in a 2 × 4 factorial arrangement was used, comprising two treatments with auxin, four with hydrogen peroxide and quercetin and 5 replicates with 48 cuttings per experimental unit, totaling 1920 cuttings per test. For the third experiment, a completely randomized in a 2 × 2 factorial arrangement was used, with two doses of auxin and two of hydrogen peroxide, combined with quercetin, and five replicates with 48 cuttings per experimental unit,

totaling 480 cuttings.

Planting of the cuttings

Cuttings were planted in a substrate composed of Canadian peat, vermiculite and rice hulls, and 4 kg m^3 single superphosphate fertilizer (SSF, P_2O_5) and slow release fertilizer, NPK (15/8/12, Basacote® Plus) supplemented with micronutrients, at 1 kg m^3 in the summer and 3 kg m^3 in winter. The substrate presented a C/N ratio in the 20-45 range.

Substrate components were blended for 20 min, and then water (20 to 30 L) were added. The substrate was placed in 55 m^3 plastic tubes. The bases of the cuttings (1 cm) were dipped in $CaSO_4$ solutions and then planted. Cuttings were transferred to the greenhouse, where they remained for 23 days at 25 - 30°C and relative humidity above 75%, by nebulizers. Water management was carried out according to the need for irrigation, controlling water saturation of the substrate. There was no need for chemical pest control because of contamination by pathogens was eliminated by the use of carefully controlled materials. After growth in the greenhouse, cuttings were moved to a shade house, where they stayed 20 days for acclimatization. Fertilization composed of macro and micronutrients was applied daily, according to cutting growth and season. After this period, the seedlings were transferred to the full sun area for other 50 days. Then, tray randomization was performed in order to ensure that seedling plants were chosen for independent analysis.

After 15-30 days of growth, a hardening fertilization was performed in which the frequency and volume of irrigation were reduced.

Seedling evaluation

Among the 48 seedlings comprising each experimental unit, 28 plant seedlings were evaluated. Periodically, at 30, 60 and 90 days, seedling height and diameter were measured and survival percentage was evaluated. Then, a destructive evaluation was performed in 20 plants, for the determination of rooting percentage, number of roots per cutting, and the average length of roots, by averaging the longest roots of each repetition.

Determination of polyamines

Polyamine content was determined according to the method proposed by Flores and Galston (1982), modified by Lima et al. (2008). Briefly, leaves and roots (0.2 g) were homogenized in 5% $HClO_4$ (3 mL). Supernatants (200 µl), saturated calcium carbonate (200 µL), and dansyl chloride (400 µL), were added in test tubes, and left 16 h in the dark at room temperature. After this period, proline (100 µL) was added. Toluene (500 µL) was used to extract dansylated polyamines. Toluene soluble extracts were applied to glass plates, coated with G60 silica gel for thin layer chromategraphy (TLC). For polyamine separation, chloroform-triethylamine (20:1, v/v) was used as solvent, and the run was monitored by UV light. After approximately 1 h, TLC plates were removed from the solvent solution and dried under air circulation. Polyamines were determined by fluorescence densitometry against standards and results were expressed as mg polyamines (spermidine, spermine and putrescine) g^{-1} fresh weight.

Determination of polyamine oxidase activity

Polyamine oxidase activity was determined according to the method reported by Stevanato et al. (1990). Leaves and root samples of *E. grandis* x *E. urophylla* (approximately 50 mg) were assayed in 20 mM Hepes buffer, pH 7.0. The reaction was performed at 30°C and followed for 7 min in a spectrophotometer (Cary 50, Varian, The Netherlands) at 555 nm. The results were expressed in ΔAbs min^{-1} g^{-1} fresh matter.

Statistical analysis

Experimental data were subjected to analysis of variance (ANOVA) and Tukey test ($p<0.05$).

RESULTS AND DISCUSSION

Effect of quercetin and IBA on cutting growth

Data obtained from the destructive evaluation of cuttings, such as rooting, root number and length, did not show statistically significant differences between treatments (Table 1). As well, regarding the effect of quercetin and IBA on plant height, diameter and survival percentage, measured at 30, 60 and 90 days after planting, the F interaction (time x quercetin x IBA) was not significantly different among treatments (Table 2 and Figure 1). For all treatments a high growth in height and diameter, high rooting and survival, including controls (Tables 1 and 2), were observed.

We concluded that other factors, such as environment, genetic material and nutrition and water conditions of donor plants, were the most important influencing factors on seedling development and rooting growth, than the application of auxin and quercetin. Other studies found that rooting can be influenced by injuries, hormonal balance, genetic constitution, presence of inhibitors and nutrition and water conditions of donor plant (Alfenas et al., 2004), and cutting development can be strongly influenced by the maturation/juvenility of seedlings and environmental conditions (Xavier et al., 2009). Conversely, positive results on seedling growth, related to flavonoids and IBA treatment, were observed in other studies. In clones of *Eucalyptus benthamii* Maiden *& Cambage dunnii* Maiden *x Eucalyptus*, the application of IBA positively influenced the processes of cutting rizogenesis, the rate of root formation and an increase of adventitious rooting (Brondani et al., 2012).

Flavonoids like quercetin, kaempferol, apigenin and other molecules, synthesized in the first process of the flavonoid biosynthetic pathway, demonstrated the ability to inhibit polar auxin transport and thereby increase the accumulation of auxin in plants (Peer and Murphy, 2007). In yerba mate (*Ilex paraguariensis*), the exogenous application of quercetin resulted in an increase of rooting percentage of 17 to 55% (Tarragó et al., 2005).

Effect of hydrogen peroxide and IBA on cutting growth and rooting

No statistically significant differences were observed

Table 1. Effect of different doses of quercetin (0, 0.25, 0.5, 1.0 g kg^{-1}) and indole butyric acid (0, 1.0 g kg^{-1}) on cutting height and diameter and survival percentage, measured at 30, 60 and 90 days after planting of *Eucalyptus grandis* x *Eucalyptus urophylla* cuttings.

Quercetin	Height (cm)		Diameter (cm)		Survival percentage (%)	
	IBA (g kg^{-1})					
	0	1.0	0	1.0	0	1.0
Quercetin (g kg^{-1})			**30 days**			
0	7.78 ± 0.34	7.61 ± 0.56	1.10 ± 0.06	1.23 ± 0.17	89.29 ± 7.58	90.00 ± 8.89
0.25	7.81 ± 0.59	7.89 ± 0.19	1.23 ± 0.17	1.17 ± 0.08	91.43 ± 6.96	88.57 ± 10.53
0.5	7.83 ± 0.30	6.99 ± 0.80	1.21 ± 0.18	1.15 ± 0.08	92.86 ± 6.19	83.57 ± 8.49
1.0	7.57 ± 0.29	7.41 ± 0.19	1.27 ± 0.16	1.21 ± 0.14	93.57 ± 6.39	95.00 ± 4.07
Quercetin (g kg^{-1})			**60 days**			
0	12.89 ± 1.04	13.11 ± 0.95	1.67 ± 0.16	1.79 ± 0.06	88.57 ± 8.89	90.00 ± 8.89
0.25	12.34 ± 1.73	15.15 ± 1.40	1.69 ± 0.19	1.82 ± 0.11	90.71 ± 5.98	88.57 ± 10.53
0.5	13.62 ± 1.49	13.51 ± 1.22	1.75 ± 0.15	1.83 ± 0.15	92.14 ± 5.87	83.57 ± 8.49
1.0	12.28 ± 1.43	13.10 ± 1.75	1.73 ± 0.15	1.74 ± 0.11	92.04 ± 6.02	93.57 ± 6.87
Quercetin (g kg^{-1})			**90 days**			
0	21.87 ± 1.03	22.55 ± 0.56	2.14 ± 0.09	2.25 ± 0.08	88.57 ± 8.89	89.29 ± 8.38
0.25	21.48 ± 2.33	24.05 ± 1.27	2.12 ± 0.14	2.35 ± 0.15	90.71 ± 5.98	88.57 ± 10.53
0.5	23.65 ± 2.18	22.70 ± 0.43	2.23 ± 0.09	2.26 ± 0.08	92.14 ± 5.87	83.57 ± 8.49
1.0	21.60 ± 2.32	22.50 ± 2.97	2.15 ± 0.15	2.26 ± 0.14	91.43 ± 5.42	93.57 ± 6.87
F	0.20		0.10		0.83	

Table 2. Effect of hydrogen peroxide (0, 1.75, 3.5, 7.0 %, v/v) and indole butyric acid (0, 1.0 g kg^{-1}) on height (cm), diameter (cm) and survival percentage, of *Eucalyptus grandis* x *Eucalyptus urophylla* cuttings, measured at 30, 60 and 90 days after planting.

Hydrogen peroxide	Height (cm)		Diameter (cm)		Survival percentage (%)	
	IBA (g kg^{-1})					
	0	1.0	0	1.0	0	1.0
H_2O_2 (%)			**30 days**			
0	7.18 ± 0.37	7.71 ± 0.40	1.16 ± 0.04	1.22 ± 0.07	92.86 ± 8.38	93.57 ± 7.74
1.75	8.03 ± 0.55	8.06 ± 0.77	1.28 ± 0.20	1.33 ± 0.22	95.00 ± 7.41	95.00 ± 5.42
3.5	7.61 ± 0.39	7.84 ± 0.81	1.24 ± 0.14	1.23 ± 0.10	97.14 ± 2.99	94.29 ± 4.07
7	7.61 ± 0.53	7.45 ± 0.30	1.22 ± 0.07	1.20 ± 0.13	95.00 ± 5.98	95.71 ± 3.91
H_2O_2 (%)			**60 days**			
0	13.93 ± 1.58	14.73 ± 1.14	1.76 ± 0.14	1.84 ± 0.07	92.86 ± 8.38	93.57 ± 7.74
1.75	15.19 ± 2.03	14.60 ± 0.94	1.86 ± 0.10	1.84 ± 0.11	95.00 ± 7.41	95.00 ± 5.42
3.5	14.09 ± 1.48	14.88 ± 1.63	1.81 ± 0.09	1.89 ± 0.10	95.71 ± 2.99	93.57 ± 3.91
7	13.98 ± 1.63	14.13 ± 1.52	1.77 ± 0.12	1.81 ± 0.09	93.57 ± 6.39	94.29 ± 6.96
H_2O_2 (%)			**90 days**			
0	24.22 ± 2.20	26.64 ± 1.47	2.25 ± 0.14	2.22 ± 0.08	92.86 ± 8.38	92.86 ± 7.14
1.75	25.52 ± 3.04	25.38 ± 1.08	2.33 ± 0.17	2.25 ± 0.13	95.00 ± 7.41	93.57 ± 6.87
3.5	23.72 ± 1.78	25.95 ± 1.60	2.17 ± 0.13	2.29 ± 0.13	95.71 ± 2.99	90.71 ± 6.96
7	24.89 ± 1.54	26.22 ± 1.04	2.22 ± 0.15	2.25 ± 0.06	93.57 ± 6.39	92.86 ± 6.96
F	0.90		0.49		0.88	

among treatments for the measured parameters, from the destructive evaluation of samples, such as rooting, root number and length (Table 3). Regarding treatments with H_2O_2 and IBA on periodically measured parameters, cutting height, diameter and percentage of survival, the F interaction (time x hydrogen peroxide x auxin) were, as well, not significantly different among treatments (Table 4).

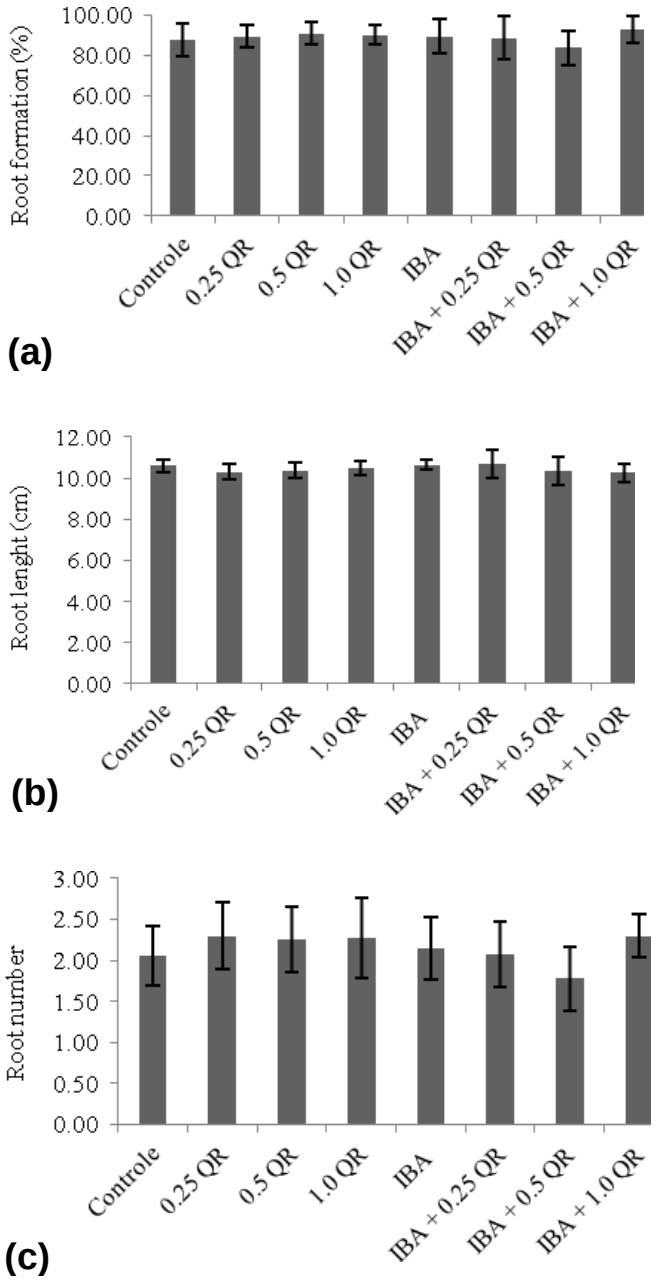

Figure 1. Effect of different doses of quercetin (0, 0.25, 0.5, 1.0 g kg^{-1}) and indole butyric acid (0, 1.0 g kg^{-1}) on (a) rooting percentage (%), (b) root length (cm) and (c) root number, measured at 90 days after planting of *Eucalyptus grandis* x *Eucalyptus urophylla* cuttings.

In the evaluation of *E. grandis x E. urophylla* samples, for all treatments, all measured parameters showed satisfactory results, including controls, and we may infer that other factors, such as environmental conditions, genetic features, and care in cutting handling were decisive for seedling development. Some authors claimed that the presence of hydrogen peroxide can affect the growth and differentiation of roots (Dunand et al., 2007; Sebastiani and Tognetti, 2004). In olive cuttings (*Olea europaea* L.), at the end of the rooting period (88^{th} day), roots number increased by 3.5% with H_2O_2 treatment in comparison to IBA treatment alone (3.40 versus 1.95 roots per cutting at 4.0 g kg^{-1} IBA and 1.80 versus 1.08 roots per cutting at 2.0 g kg^{-1} IBA) (Sebastiani et al., 2002). However, our results showed no changes in rooting according to the levels of peroxide. Probably, other factors, such as genetic and environmental conditions may influence the development of *E. grandis x urophylla* samples, and no appa-

Table 3. Effect of the addition of quercetin (0.5 g kg^{-1}), associated with hydrogen peroxide (3.5 %), and indole butyric acid (1.0 g kg^{-1}) on seedling height (cm), diameter (cm) and survival percentage, measured at 30, 60 and 90 days after planting of *Eucalyptus grandis* x *Eucalyptus urophylla* cuttings.

Quercetin + hydrogen peroxide	Height (cm)		Diameter (cm)		Survival (%)	
	IBA (gkg^{-1})					
	0	1.0	0	1.0	0	1.0
Quercetin (g kg^{-1}) + H_2O_2 (%)	**30 days**					
0.0 + 0.0	7.09 ± 0.39	7.15 ± 0.36	0.83 ± 0.12	0.85 ± 0.07	90.71 ± 4.07	90.71 ± 5.42
0.5 + 3.5	7.60 ± 0.52	7.03 ± 0.41	0.87 ± 0.07	0.84 ± 0.07	85.71 ± 4.37	90.00 ± 8.89
Quercetin (g kg^{-1}) + H_2O_2 (%)	**60 days**					
0.0 + 0.0	11.59 ± 0.73	11.97 ± 1.32	1.62 ± 0.06	1.71 ± 0.07	76.43 ± 11.18	75.71 ± 7.32
0.5 + 3.5	12.63 ± 1.57	11.99 ± 0.77	1.62 ± 0.18	1.64 ± 0.09	72.86 ± 7.41	72.14 ± 5.30
Quercetina (g kg^{-1}) + H_2O_2 (%)	**90 days**					
0.0 + 0.0	16.28 ± 0.78	16.84 ± 1.81	1.94 ± 0.13	2.04 ± 0.13	71.43 ± 7.58	71.43 ± 6.68
0.5 + 3.5	18.15 ± 2.35	17.05 ± 0.71	2.13 ± 0.18	2.01 ± 0.12	72.14 ± 9.24	70.00 ± 6.49
F	0.59		0.28		0.78	

Table 4. Content of polyamines (putrescine, spermidine and spermine, expressed in µg g^{-1} fresh tissue) in leaves and roots of *Eucalyptus grandis* x *Eucalyptus urophylla* seedlings treated with different combinations of hydrogen peroxide (3.5 %), quercetin (0.5 g kg^{-1}) and indole butyric acid (1.0 g kg^{-1}).

Leaf and root	Quercetin + hydrogen peroxide	Putrescine		Spermidine		Spermine		Polyamine ratio Put/(Spd+Spm)	
		IBA (g kg^{-1})							
	H_2O_2 (%) + quercetin (g kg^{-1})	0	1.0	0	1.0	0	1.0	0	0.0
Leaves	0.0 + 0.0	5.57 ± 0.45	5.33 ± 0.31	5.68 ± 0.05 bA	8.17 ± 0.18 aB	8.87 ± 0.53 aA	9.09± 0.60 aA	0.38	0.31
	3.5 + 0.5	6.13 ± 0.39	6.13 ± 0.64	3.79 ± 0.14 bB	8.90 ± 0.62 aA	9.21 ± 1.45 aA	6.43± 0.17 bB	0.47	0.40
	CV (%)	8.02		4.98		9.91			
Roots	0.0 + 0.0	7.29 ± 0.33 aA	4.99 ± 1.03 bA	6.66 ± 0.65	2.94 ± 0.23	13.38 ± 0.59	12.94 ± 0.73	0.36	0.31
	3.5 + 0.5	1.66 ± 0.43 bB	4.41 ± 1.28 aA	6.98 ± 1.31	2.69 ± 0.44	16.16 ± 1.64	13.68 ± 0.18	0.07	0.29
	CV (%)	18.83		21.42		6.76			

*Lowercase letters compare means between treatments with indole butyric acid. Capital letters compare levels of hydrogen peroxide between each concentration of auxin. Means followed by the same letter are not statistically different by the Tukey test at 5% probability

rent effect was noticed with the applied substances (Figure 2).

Effect of quercetin, hydrogen peroxide and IBA on development of *Eucalyptus* cuttings

An important reduction of the percentage of survival at 30 and 60 days after planting was observed (Table 2), which was attributed to the moving of seedlings from the shaded house to the full sun for hardening and acclimatization, occurring in May, characterized by the reduction of daily light and temperature, which hindered the establishment of seedlings. At the same time a low development of seedling height, diameter, root length and number was observed, when compared to previous experiments. This effect could be attributed to the different period during the year: the first two experiments were carried out from October to the end of December, while the second experiment was performed in March, ending in May. The last one has fewer daily light hours, and lower temperature and rainfall, which may have hampered the development of the seedlings. Despite these lower

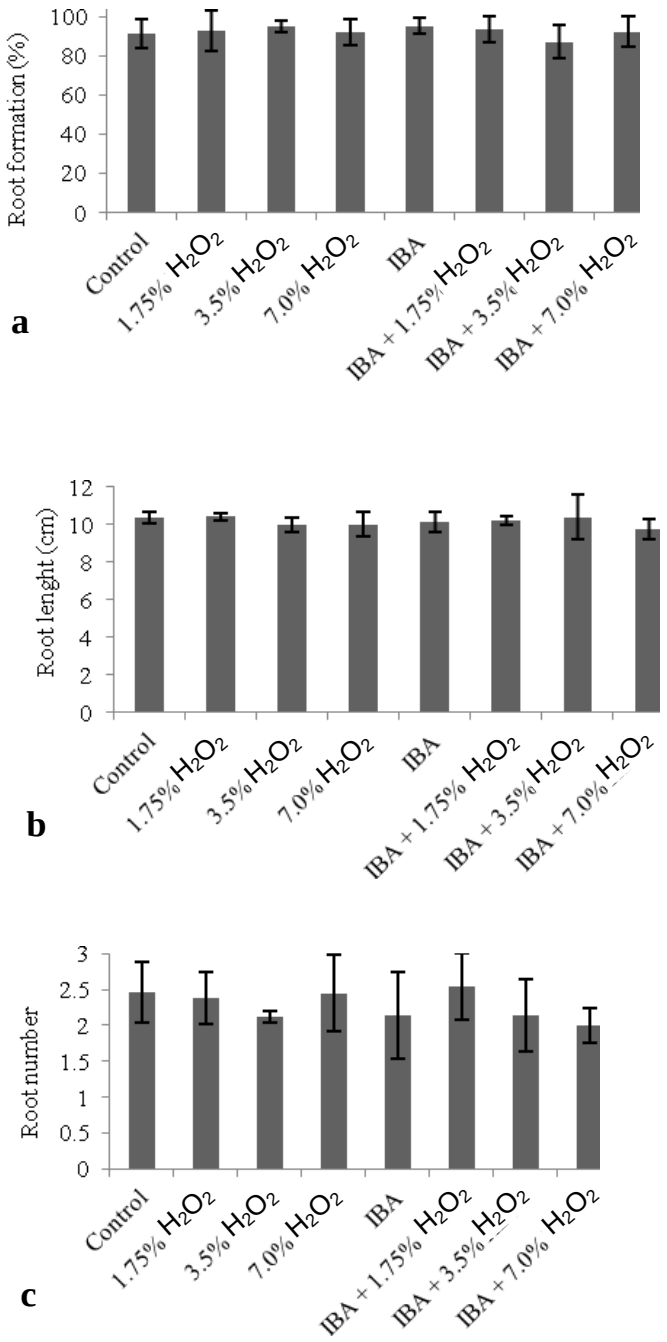

Figure 2. Effect of hydrogen peroxide (0, 1.75, 3.5, 7.0%, v/v) and indole butyric acid (0, 1.0 g kg^{-1}) on (a) root formation (%), (b) root length (cm) and (c) root number, measured at 90 days after planting of *Eucalyptus grandis* x *Eucalyptus urophylla* cuttings.

results, biometric variables during experiments showed a good performance, which emphasizes the influence of genetic factor for successful planting. The doses of auxin and quercetin, associated with hydrogen peroxide, and their combinations, showed no influence on sample development. In other studies, positive results were found. An effect of IBA on rooting of hardwood cuttings of several species, such as fig (*Ficus carica*) (Pio et al., 2003) and blueberry (*Vaccinium ashei*) (Vignolo et al., 2012) has been reported. The application of quercetin increased rooting rate of 17 to 55% in yerba mate (Tarrago et al., 2005). Hydrogen peroxide has also been described as effective in root forming in cuttings of olive (*Olea europaea* L.), favoring both the formation of roots and increasing the number of roots per cutting (Sebastiani and Tognetti, 2004) (Figure 3).

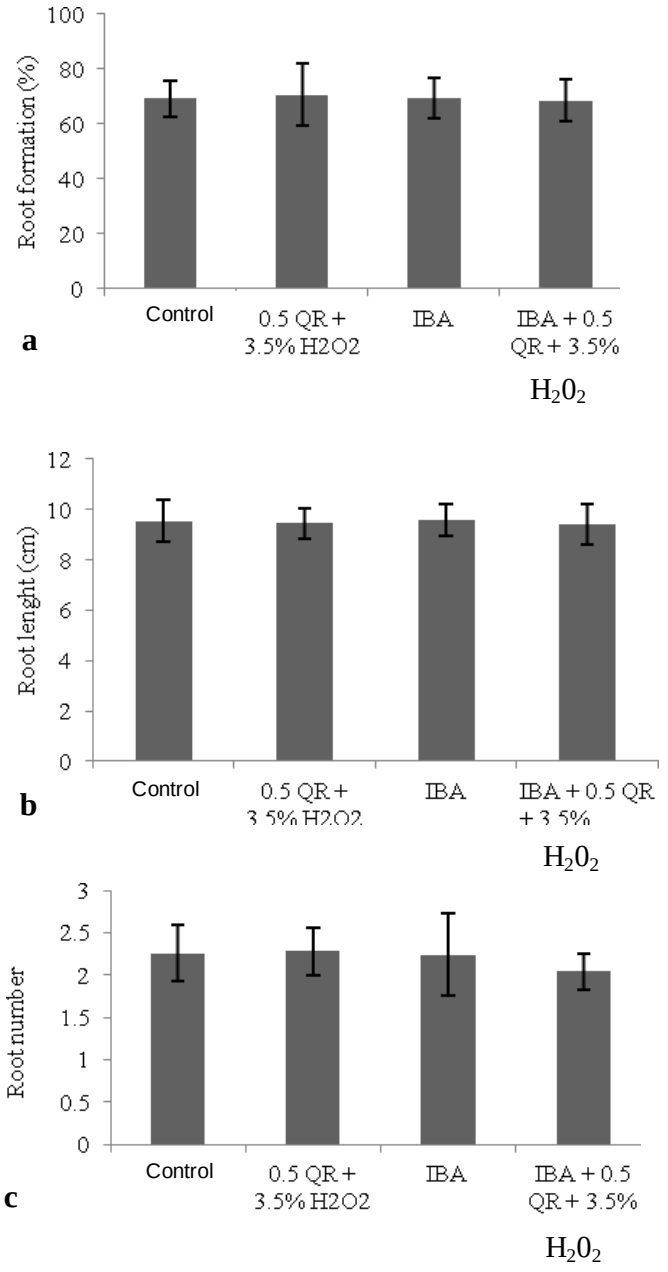

Figure 3. Effect of the addition of quercetin (0.5 g kg^{-1}), associated with hydrogen peroxide (3.5 %), and indole butyric acid (1.0 g kg^{-1}) on (a) root formation (%), (b) root length (cm) and (c) root number, measured at 90 days after planting of *Eucalyptus grandis* x *Eucalyptus urophylla* cuttings

Biochemical assessments

In the present study, no effect on rooting and development of *Eucalyptus* seedlings were observed, thus changes found in the levels of polyamines and in their oxidation enzymes should be due to the effect of substances used during the treatments. No interaction effects (quercetin x auxin x hydrogen peroxide) were observed on the diamine putrescine (Put) in leaves, thus the effects of the factors were separated (Figures 4A and 4B). An increase in the content of putrescine in cuttings treated with quercetin, associated with H_2O_2, was observed (Figure 4B). Similar results were found for spermidine (Spd), either in the presence or absence of IBA, whereas decreased levels of spermine (Spm) were determined in the presence of a combination of IBA, hydrogen peroxide and quercetin (Table 4).

A reduction in the levels of putrescine, compared to

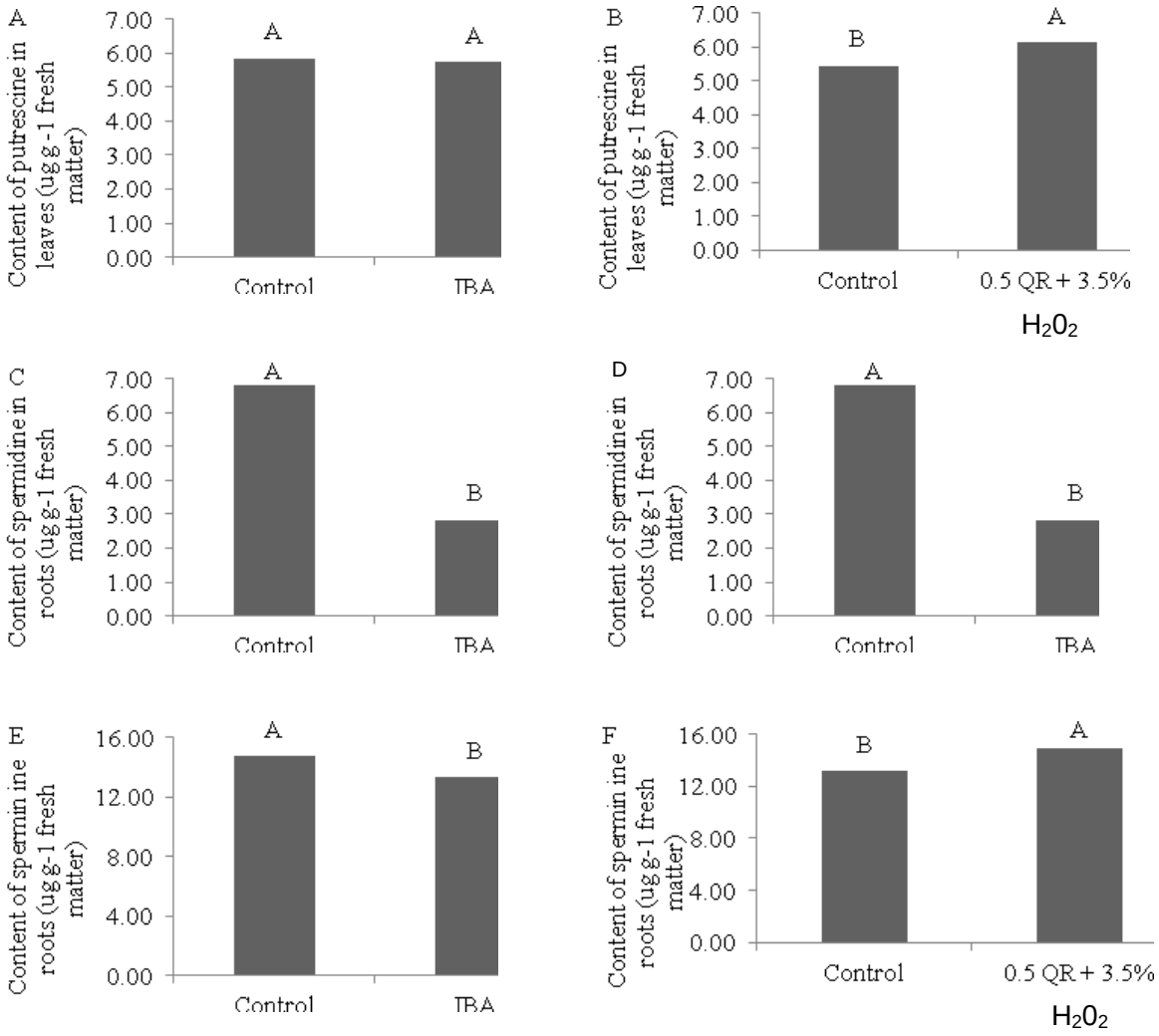

Figure 4. Content of putrescine in leaves (A and B), spermidine (C and D) and spermine (E and F) in roots of seedlings of *Eucalyptus grandis* x *Eucalyptus urophylla* treated with different combinations of hydrogen peroxide (3.5%), quercetin (0.5 g kg^{-1}) and indol butyric acid (1.0 g kg^{-1}).

controls, was observed in roots for all treated samples (Table 4). The polyamines spermidine and spermine showed no interaction among used substances. The levels of both polyamines were reduced by the action of IBA (Figures 4C and 4D). The combination of hydrogen peroxide and quercetin did not alter the levels of spermidine (Figure 4D) and caused an increase of spermine content (Figure 4F). According to Su et al. (2006), the levels of agmatine (Agm), spermidine and spermine are positively related to root development, while the content of putrescine has a neutral or negative effect. Since all parameters related to growth and development were considered as satisfactory, it is possible that the clone used in the present report provided sufficient levels of polyamines, or that these plants are not responsive to treatments used due to insufficient levels of polyamines.

It has been reported that these substances are directly correlated with some specific phases of cell division (Chattopadhyay et al., 2002). The lower concentration of polyamines in roots treated with quercetin, hydrogen peroxide and IBA (Table 4) than controls, may be related to a diminished capacity to synthesize putrescine, or alternatively, to the increased activity of polyamine oxidase (PAO), as documented (Table 5). This last result, evidencing a high catabolism of putrescine, was already described in young roots (Paschalidis and Roubelakis-Angelakis, 2005).

Generally, in old tissues, a decrease in level of auxin is negatively correlated with the activity of amino oxidases (Cona et al., 2003). Thus, clones treated with auxin would present higher levels of polyamines and lower activities of PAOs. In the present study, no differences due to the addition of IBA on leaves were found. We observed a different effect in roots: i.e., the combined treatment with hydrogen peroxide, quercetin and auxin induced a decrease of the activity of polyamine oxidase. With a

Table 5. Polyamine oxidase activity (expressed as ΔAbs $min^{-1}g^{-1}$fresh weight) in leaves and roots of *Eucalyptus grandis* x *Eucalyptus urophylla* seedlings treated with different combinations of hydrogen peroxide (3.5 %), quercetin (0.5 g kg^{-1}) and indole butyric acid (1.0 g kg^{-1}).

Parameter	Quercetin + hydrogen peroxide	Polyamine oxidase activity	
		IBA (g kg^{-1})	
	H_2O_2 (%); Quercetin (g kg^{-1})	0	1.0
Leaves	0.0 + 0.0	2.08 ± 0.15	2,37 ± 0,42
	3.5 + 0.5	2.38 ± 0.20	2,38 ± 0,40
	CV (%)	13.79	
Roots	0.0 + 0.0	0.82 ± 0.06 aA	0,91 ± 0,13 aA
	3.5 + 0.5	0.87 ± 0.11 aA	0,71 ± 0,03 bB
	CV (%)	10.96	

*Lowercase letters compare means between treatments with indole butyric acid. Capital letters compare levels of hydrogen peroxide between each concentration of auxin. Means followed by the same letter are not statistically different by the Tukey test at 5% probability.

concomitant increase of putrescine content, which can also be observed from the value of polyamine ratio (Put/(Spd + Spm) = 0.29) (Table 4).

No statistically significant differences were found in the activity of polyamine oxidase in leaves (Table 5). Differently, in roots of *Eucalyptus* seedlings, the activity of PAO was reduced upon treatment with quercetin alone and associated with IBA and hydrogen peroxide. Several reports showed the involvement of PAO on organism development and stress responses (Moschou et al., 2013).

Polyamine oxidases are responsible for the degradation of polyamines, which in turn influence different aspects of root development, such as an increased meristematic activity, leading to root elongation, lateral and adventitious root formation (Couee et al., 2004). However, this effect cannot be ascribed in the present study, since no alterations were observed with treatments. Generally, a low rate of polyamine catabolism occurs in young and growing leaves, as well as in apices (Paschalidis and Roubelakis-Angelakis, 2005). The lowest PAO activity was found in roots treated with hydrogen peroxide + IBA + quercetin, and it can be attributed to the presence of auxin, because low levels of this regulator have been associated with the expression of genes which induce the formation of PAO (Chen et al., 2001).

Conclusion

Cuttings of a clone (CL1) from *E. grandis* x *Eucalyptus urophylla* were treated with hydrogen peroxide in combination with quercetin or with indole butyric acid, and rooting and developmental parameters were evaluated. The clone was unresponsive to treatments used in relation to seedling height, diameter, survival, rooting percentage, root length and number of roots per cutting. Treatments induced a decrease of the levels of putrescine and of the activity of PAO in roots. The absence of positive responses, the use of these substances as a treatment to improve seedling production is economically unviable. We suggest that in a future a research focusing on other bioactive ingredients.

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Effects of plant growth regulators on *in vitro* cultured nodal explants of cassava (*Manihot esculenta* Crantz) clones

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Cassava (*Manihot esculenta* Crantz) is one of the major food security crops in Ethiopia. Recently, clean planting materials of improved cassava cultivars are in high demand. A limitation, however, is the low multiplication ratio (1:10) of the crop *via* conventional methods. Thus, a study was undertaken to develop an efficient *in vitro* mass propagation protocol for two elite cassava clones, 44/72-NR and 44/72-NW. Combination of different plant growth regulators (PGRs); four concentrations of 6-benzylaminopurine (BAP) and kinetin (Kin) on shoot multiplication and that of α-naphthaleneacetic acid (NAA) and BAP each at four concentration combination on root induction were assessed. The experiments were factorial laid out in a completely randomized design (CRD) with PGRs as one-factor and clones as another, replicated five times. Significant ($p< 0.05$) interaction effects were observed in response to shoot multiplication and root induction treatments within six weeks of culture. Murashige and Skoog (MS) medium containing BAP and Kin each at 0.75 mg/L gave an average of 7.30 shoots per explant than other media combinations. Consecutively, the regenerated cassava shoots produced an average of 6.14 roots within four weeks in a 0.5 mg/L NAA medium and were successfully acclimatized and transferred to field.

Key words: Cassava, *Manihot esculenta*, clone, *in vitro,* nodal bud, plant growth regulators.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a major food crop for about 800 million people in sub-Saharan Africa and other parts of the world (Taye, 2009). Although reliable statistical information on the area of production and productivity of cassava in Ethiopia is lacking, the crop had long been in cultivation particularly in the Southern, South Western and Western parts of the country (IAR, 1981). In all those growing regions, the farmers are paying more attention to the crop due to its being reliable and cheap source of food available year round. In addition, it is considered as the only flexible and alternative crop to the poor farmers due to its acceptable yield

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under marginal soil and drought prone conditions with minimum inputs (FDRE, 2002). Despite its great potential to attain food security, cassava has gained little attention in the national agricultural research system of Ethiopia. However, a remarkable growth has been registered in its production within the last decade compared with other crops (Amsalu, 2003).

Most recently, the Ethiopian government has considered cassava as one of the major food security crops. It has also been given top research priority with special emphasis on its promotion and expansion to all drought prone and degraded regions of the country (FDRE, 2002). Consequently, there is an urgent need for disease free and high quality cassava planting materials in the national production system. Towards this aim, Hawassa Agricultural Research Center (HaARC) has released two cassava varieties, *Qulle* and *Kello* for the Southern region (HaARC, 2008) and Jimma Agricultural Research Center (JARC) is about to release a number of varieties for the South Western parts of the country. The limitations, however, are the low multiplication ratio (1:10) of the crop using traditional production methods and systemic infections, which hamper the progress in replacing susceptible or non-adapted varieties, or in expanding cassava into new areas. Thus, tissue culture techniques could be a feasible option to overcome these problems.

Plant tissue culture techniques were developed five decades ago (Santana et al., 2009) and since then, they have been recognized as powerful tools for studying and solving basic and applied problems of cassava production and productivity like many other crops (Robert and Dennis, 2000; Adane, 2009). This technique is faster and requires less space than that required for conventional methods of preparing cuttings (Loyola-Vargas and Vazques-Flota, 2006). In this regard, available research findings (Were et al., 2004; Le et al., 2007) have long proved the tissue culture techniques to be the only realistic and efficient means for supplying large volumes of true-to-type clean planting materials of any new high-value crop variety like cassava within short period. However, multiplication of tissues requires the optimization of the plant growth regulators (PGRs) concentrations in the MS media and these PGR requirements vary with species since PGRs often determine the course of morphogenesis (Staden et al., 2008; IITA, 2009). Earlier, Konan et al. (2006) and Acedo (2009) revealed that shoot multiplication of many crops including cassava could be enhanced with a relatively higher concentration of cytokinins (disrupt apical dominance of the shoot tips); while rooting is promoted by the use of auxins. Kane (2005) also reported cytokinins, 6-benzylaminopurine (BAP) and kinetin (Kin) and auxin, α-naphthaleneacetic acid (NAA) as the most widely used and effective (0.01-10 mg/L) PGRs for shoot multiplication and root induction, respectively.

Smith et al. (1986) and Konan et al. (1997) were the first to report success in the production of an average of 5 to 6 shoots per bud from *in vitro* culture of cassava, although the field survival rates recorded were low (62 - 74%). Several workers have since these earlier studies also succeeded in *in vitro* nodal culture of cassava (Konan et al*.,* 2006; Medina et al., 2006; Escobar et al., 2009). Despite these reported successes, no work has so far been done to develop an *in vitro* mass propagation protocol for those elite cassava clones in Ethiopia, and development of such a protocol is urgently needed to ensure rapid mass propagation and dissemination of the improved cassava clones to respond to the prevailing high interest to increase cassava production and introduce it into new potential areas of the country. In view of all these, the objective of this study was to develop an efficient *in vitro* mass propagation protocol for two elite cassava clones, 44/72-NR and 44/72-NW.

MATERIALS AND METHODS

Study location

The research work was conducted in the Tissue Culture Laboratory of the Plant Biotechnology Division, Jimma Agricultural Research Centre (JARC), Jimma, Ethiopia. The center is located 363 km south west of the capital, Addis Ababa at 7°46' N latitude and 36°0'E longitude.

Genetic materials

Two preferred cassava clones, 44/72-NR and 44/72-NW, obtained from JARC were selected due to their best performance across the South Western region of Ethiopia with respect to early maturity, high productivity, and resistance to diseases and pests compared to other cultivars tested.

Stock plant establishment

Twenty-five to thirty cm long mature stem cuttings with 5-8 nodes of both clones were taken from the horticulture research field of JARC and soaked in a 0.3% (w/v) Kocide-101 solution for five minutes followed by rapid rinsing under running tap water, twice. The cuttings were planted in plastic pots of 2 L volume filled with a pre-sterilized potting mix of forest soil, well-decomposed coffee husk, and red sand at the respective ratio of 1:1:2 by volume (JARC experience) and the stock plants were established in the maintenance greenhouse of the biotechnology division at an average temperature of 25 ± 2°C.

Explant sterilization and initiation

Three-to-five-week old sprouted nodal buds of 1.5 - 2.0 cm length were collected from both clones, washed thoroughly once under running tap water using a liquid detergent and kept soaked in a 0.3% (w/v) Kocide-101 solution for 30 min followed by rapid rinsing three times with double distilled water in the laminar flow hood cabinet. The nodal buds were then soaked in 70% (v/v) ethyl alcohol for 1 min and immediately rinsed three times with double distilled water before the actual sterilization with sodium hypochlorite (NaOCl). After disinfection in 0.1% (v/v) NaOCl with 1-3 drops of Tween-20 (wetting agent) for 10 min and washing with double distilled water thrice, all the dead and chlorine affected tissues of the nodal bud explants were removed under aseptic conditions.

The nodal buds were then placed in test tubes containing solid Murashige and Skoog (MS-1962) medium supplemented with 100 mg/L myo-inositol, 1 mg/L thiamine, 1.6 mg/L GA_3, 0.01 mg/L NAA with 3% (w/v) sucrose (carbon source) and 0.8% (w/v) agar (gelling agent) at 5.8 pH (IITA, 2009) for initiation. The test tubes were incubated at 23-24°C under 16/8 h light/dark cycles and a light intensity of 1000 Lux. The nodal buds of the two clones normally took 1-2 months to develop into a plantlet ready for sub culture.

Treatments and experimental design

The initiated 4-8 weeks old plantlets from the two clones were multiplied through stem cuttings containing single node. The nodal buds were placed onto full strength solid MS basal medium in Magenta culture vessels containing different concentrations and combinations of PGRs namely 6-Benzylaminopurine (BAP) and Kinetin (Kin) each at four concentrations (0, 0.75, 1.5, and 2.25 mg/L) in all combinations for shoot multiplication and α-naphthalene acetic acid (NAA) at four concentrations (0, 0.5, 1, and 1.5 mg/L) in all combination with BAP at four concentrations (0, 2.5, 5, and 7.5 mg/L) for root induction (Table 1). Before conducting the rooting experiment, the initiated mini *in vitro* derived shoots were transplanted onto a fresh growth regulator free MS basal medium for five weeks in order to avoid any carry over effect of NAA used at the initiation stage.

The two clones were equally and randomly treated while all exogenous factors were held constant except the factors being considered. In all cases, the brim of each test tube and/or Magenta culture vessel was flamed together with their caps prior to closing and sealed with a strip of Para film. The vessels were clearly labelled with the media code, date of inoculation as well as name of variety and incubated at 25 ± 2°C, 16 h photoperiod with a light intensity of 2000-3000 Lux from cool white 40 watt florescent bulbs. Completely Randomized Design (CRD) in factorial arrangement with PGRs as one-factor and clone as another factor with five replications and five nodal buds per replication was used. In order to ensure reliability of the results, the experiments in the study were repeated twice.

Acclimatization and transfer of plantlets

After the plantlets had produced 3-5 leaves and initiated roots within four weeks, they were removed from the glass jar and transferred to a container of warm (10°C) double distilled water and gently rinsed to remove the agar-media off the roots followed by immersion into a 0.3% (w/v) Kocide-101 solution (prophylaxis measure). Finally, 30 plantlets (15 plantlets from each clone) were planted in six plastic pots of 2 L volume filled with a pre-sterilized potting mix of forest soil, well-decomposed coffee husk, and red sand at the respective ratio of 1:1:2 by volume (JARC experience) and acclimatized in a 70% shade netted greenhouse for two successive weeks followed by 30% shaded greenhouse for a week and then transferred to the environment with ambient conditions.

Data collected

After 2-3 weeks, the plantlets were studied for various growth analysis parameters and transferred to green house. During the research work, data on shoot length (cm), number of nodes/plantlet, number of leaves/plantlet, number of shoot/plantlet, number of roots/plantlet, root length (cm)/plantlet, shoot fresh and dry weights (g) as well as root fresh and dry weights (g) and root to shoot weight ratio were recorded on 1600 samples taken from shooting and rooting experiments each consisting of 800 samples.

Data analysis

The average data estimated from the raw data collected for each trait in the two experiments were subjected to analysis of variance (ANOVA) using statistical analysis software (SAS), version 9.2. Log and arcsine transformation techniques were applied for all counted and percentage data of each trait respectively, in order to fulfil the assumptions of ANOVA (Montgomery, 2005) and mean comparesons were undertaken according to Student-Newman-Keuls multiple-range test (SNK) at the alpha level of 5%.

RESULTS AND DISCUSSION

Effects of cytokinins on *in vitro* shoot multiplication of cassava

Different concentrations and combinations of cytokinins, BAP and Kin, were tested to compare their effectiveness on shoot regeneration of cassava using axillary nodal bud culture. Shoot regeneration of the two cassava clones were influenced differentially by the concentrations and combinations of the cytokinins used (Table 2). Both BAP and Kin had a significant ($P \leq 0.01$) effect on the number of shoots, leaves, and nodes, as well as shoot length, shoot fresh and dry weights whilst only leaf number was significantly ($P \leq 0.05$) affected by clone type (Table 2). Similarly, all shoot growth and development parameters considered were significantly ($P \leq 0.01$) affected by BAP x Kin interaction. The effect of interaction between BAP and clone type on shoot fresh and dry weight parameters was also significant ($P \leq 0.01$). However, Kin × clone and BAP × Kin × clone interactions had no significant effects on all the shoot multiplication parameters evaluated.

The full strength solid MS basal medium supplemented with BAP and Kin combination (both at 0.75 mg/l) resulted in regeneration of the highest mean maximum values on almost all shoot growth parameters considered namely, number of shoots (7.30), leaves (5.67), and nodes (5.65), shoot length (5.05 cm), shoot fresh (2.55 g), as well as dry (2.20 g) weights per explant (Table 3). This could be associated with the synergistic effect of the two cytokinins when combined, and their effectiveness even at low concentrations as earlier reported (Onuoch and Onwubiku, 2007; Staden et al., 2008) in cassava. Both authors observed similar effects of the two cytokinins when combined at concentrations as high as 0.75-1 mg/L. Growth and development inhibitory effect of the two cytokinins were reported when they were combined at very high concentrations (Berrie, 1984; FFTC, 2009) in cassava, which corroborates with our present observation. In general, combining BAP and Kin (both above 0.75 mg/L) has no additional advantage as almost all of the evaluated shoot growth parameters failed to improve at higher concentrations and combinations of the two cytokinins (Table 3).

In accordance with the results of this study, previous reports in cassava (Kartha et al., 1984; Ogburia, 2003; Konan et al., 2006) and Egyptian sweet potato (El Far et

Table 1. Shoot multiplication and rooting treatment combinations arrangement.

Experiment type	Hormone Source (mg/L)	Treatment combination															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Shoot Multiplication	BAP	0	0	0	0	0.75	0.75	0.75	0.75	1.5	1.5	1.5	1.5	2.25	2.25	2.25	2.25
	Kin	0	0.75	1.5	2.25	0	0.75	1.5	2.25	0	0.75	1.5	2.25	0	0.75	1.5	2.25
Root regeneration	BAP	0	0	0	0	2.5	2.5	2.5	2.5	5	5	5	5	7.5	7.5	7.5	7.5
	NAA	0	0.5	1.0	1.5	0	0.5	1.0	1.5	0	0.5	1.0	1.5	0	0.5	1.0	1.5

Table 2. Mean square estimates of *in vitro* induced shoot parameters of cassava explants as affected by different concentrations and combinations of BAP, Kin and clone (ANOVA).

Treatment	DF	Shoot multiplication parameters/explant					
		Shoot				Number of	
		Number (No)	Length (cm)	Fresh Wt (g)	Dry Wt (g)	Leaves (No)	Nodes (No)
BAP	3	2.665**	3.602**	1.600**	1.379**	2.670**	1.302**
Kin	3	1.28**	0.680**	0.218**	0.252**	0.425**	0.225**
BAP x Kin	9	0.52**	0.479**	0.171**	0.150**	0.386**	0.098**
Clone	1	0.01	0.021	0.001	0.007	0.028*	0.530
BAP x Clone	3	0.04	0.009	0.042**	0.029**	0.049	0.016
Kin x Clone	3	0.04	0.024	0.001	0.001	0.007	0.002
BAP x Kin x Clone	9	0.09	0.015	0.004	0.004	0.010	0.014
R^2 (%)		77.0	89.8	85.9	88.9	88.5	73.1

*, ** indicates that Mean square values of shoot parameters considered are significant at 0.05 or 0.01 probability level, respectively.

al., 2009) showed a reduction of shoot growth characters in response to in vitro culture when BAP and Kin were combined at higher concentrations.

Significant ($P < 0.01$) differences were observed between the two clones for the number of leaves regenerated (Supplementary Data). The mean number of leaves regenerated per explant from clone 44/72-NR was 7% higher than the number of leaves regenerated from clone 44/72-NW. This variation in number of leaves between the clones could be associated with the variation in the amount of the endogenous cytokinins available in the explant buds rather than the effect of the externally applied ones. In agreement with the results of the present study, Escobar et al. (2009) reported that variations between clones in their response to similar treatments were primarily due to the presence of variable levels of cytokinin in explant buds.

Moreover, the leaves produced from clone 44/72-NR were morphologically good looking and had deep green colour than of 44/72-NW (Figure 1). This variation in the colour of the regenerated leaves might be due to the two cytokinins which enhanced chlorophyll development in the leaves of clone 44/72-NR as compared with the leaves of clone 44/72-NW, which also suggests that chlorophyll development at least in these two cassava clones is controlled by different genes. Consistent with this result, Konan et al. (2006) and Wondwosen (2009) also reported differential responses of different cassava clones *in vitro* for number of leaves regenerated.

As shown in Figure 2, the highest mean shoot fresh (1.94 g) and dry (1.56 g) weights per explant were observed in both clones on MS medium supplemented with 0.75 mg/L BAP. This shows the effectiveness of BAP at lower concentration in inducing axilary shoot proliferation and development of chlorophyll and confirms the report of Kulaeva et al. (2002) which demonstrated that the enhanced development of etioplasts especially grana in response to low BAP could accelerate the photosynthetic rate and thereby influence the shoot fresh and dry weights of the two clones. The result also corroborates the earlier submission by Najma and Uzman (2001) who reported the potential of BAP to elicit a maximum response at appreciably lower concentrations.

Effects of auxin and cytokinin on *in vitro* root induction of cassava

In this experiment, different concentrations and combinations of NAA and BAP were tested to compare their effectiveness on root regeneration of cassava. All the characters evaluated were found to be influenced by the different concentrations and combinations of the NAA and BAP applied. The number of main roots, root length, root fresh and dry weights, and root to shoot weight ratio

Table 3. Interaction effect of BAP and Kin on shoot growth and development of cassava explants after six weeks of *in vitro* culture.

Cytokinin source		Growth parameters/explants					
		Shoot				Number of	
BAP (mg/L)	Kin (mg/L)	Number (No)	Length (cm)	Fresh weight (g)	Dry weight (g)	Leaves (number)	Nodes (number)
0.00	0.00	1.60^{k}	1.53^{f}	0.55^{g}	0.36^{gh}	2.33^{f}	2.34^{fg}
	0.75	4.60^{c}	3.82^{b}	1.70^{b}	1.25^{c}	3.10^{d}	3.18^{c}
	1.50	3.90^{de}	3.82^{b}	1.51^{c}	1.04^{d}	2.74^{e}	2.70^{de}
	2.25	3.10^{fg}	3.09^{d}	1.30^{d}	0.84^{e}	2.40^{f}	2.38^{ef}
0.75	0.00	5.40^{b}	3.69^{b}	1.81^{b}	1.42^{b}	4.02^{b}	3.61^{b}
	0.75	7.30^{a}	5.05^{a}	2.55^{a}	2.20^{a}	5.67^{a}	5.65^{a}
	1.50	4.10^{d}	3.41^{c}	1.55^{c}	1.08^{d}	3.29^{c}	3.38^{bc}
	2.25	3.20^{f}	3.12^{d}	1.24^{d}	0.80^{e}	2.84^{e}	2.86^{d}
1.50	0.00	3.90^{de}	3.53^{c}	1.42^{c}	0.98^{d}	2.72^{e}	2.76^{de}
	0.75	3.70^{e}	3.04^{d}	1.05^{e}	0.65^{f}	2.83^{e}	2.84^{d}
	1.50	2.90^{fgh}	2.11^{e}	0.78^{f}	0.43^{g}	2.21^{fg}	2.24^{fgh}
	2.25	2.40^{i}	1.55^{f}	0.68^{f}	0.34^{gh}	2.08^{gh}	2.10^{fgh}
2.25	0.00	2.80^{gh}	1.97^{e}	0.50^{gh}	0.25^{hi}	2.13^{gh}	2.12^{fgh}
	0.75	2.60^{hi}	1.37^{f}	0.42^{ghi}	0.20^{i}	1.98^{hi}	1.97^{fgh}
	1.50	2.00^{j}	0.93^{g}	0.37^{hi}	0.16^{i}	1.88^{i}	1.88^{gh}
	2.25	1.80^{jk}	0.65^{h}	0.31^{i}	0.12^{i}	1.80^{i}	1.82^{h}
CV (%)		9.84	8.05	8.14	6.97	6.68	6.00

Means followed by the same letter within a column are not significantly different at 5% probability level, according to Student-Newman-Keuls multiple-range test (SNK).

characters were significantly (P ≤ 0.01) affected by both NAA and BAP (Table 4).

Similarly, there was significant (P ≤ 0.01) difference between the two clones for all the root growth parameters evaluated except root fresh weight and root to shoot weight ratio. Also, NAA × BAP interaction had significant (P ≤ 0.01) effect on all root growth parameters examined. The interaction effect of NAA and clone was observed to be significant (P ≤ 0.05) only on main root length, while the effect of BAP × clone interaction had non-significant effect on all parameters considered. Moreover, only main root length was significantly (P ≤ 0.01) influenced by NAA × BAP x clone interactions.

The lower auxin-cytokinin combination (0.5 mg/l NAA and 2.5 mg/L BAP) led to a considerable increase in the mean main root number (3.86), root fresh (3.57 g), and dry (2.08 g) weights per explant (Table 5). Likewise, the maximum mean root to shoot weight ratio (3.04) per explant was recorded in 1.0 mg/L NAA and 2.5 mg/L BAP combination enriched medium. This could be associated with the synergistic effect of auxin and cytokinin, when both were applied together at lower concentrations. This is in line with the earlier submission by Staden et al. (2008) who reported synergistic effect of NAA and BAP in cassava when combined at lower concentrations. Also, treatment combinations of 1.5 mg/L NAA with 5.0 and 7.5 mg/L BAP resulted in significant (P > 0.05) reduction in mean root fresh and dry weights (gm) per explant, respectively. Previously, Konan et al. (2006) and Zimmermann et al. (2009) found an antagonistic effect of higher BAP on NAA activity on root induction of selected cassava clones, which corroborates the results of the present study.

On the other hand, the shoots of both clones cultured on MS medium with 0.5 mg/L NAA alone had numerous long main roots and very few secondary roots (Figure 3). This is a well documented unique effect of auxin (NAA) that regulates numerous developmental processes such as tissue swelling, cell division, cell elongation, and formation of adventitious roots when it is fortified at lower concentrations (Wilmoth et al., 2005). From the results of this study, it was observed that combining auxin and cytokinin in root induction medium has no additional benefit since NAA *per se* is enough to achieve the required results. Similar findings with this result were reported previously in cassava (Ogburia, 2003; Le et al., 2007; Escobar et al., 2009; Wondwosen, 2009) and Egyptian sweet potato (El Far et al., 2009).

Significant (P ≤ 0.01) variations among the two clones were observed for the number of main root and root dry weight characters. Clone 44/72-NR produced the maximum mean number of main root (2.86) and root dry

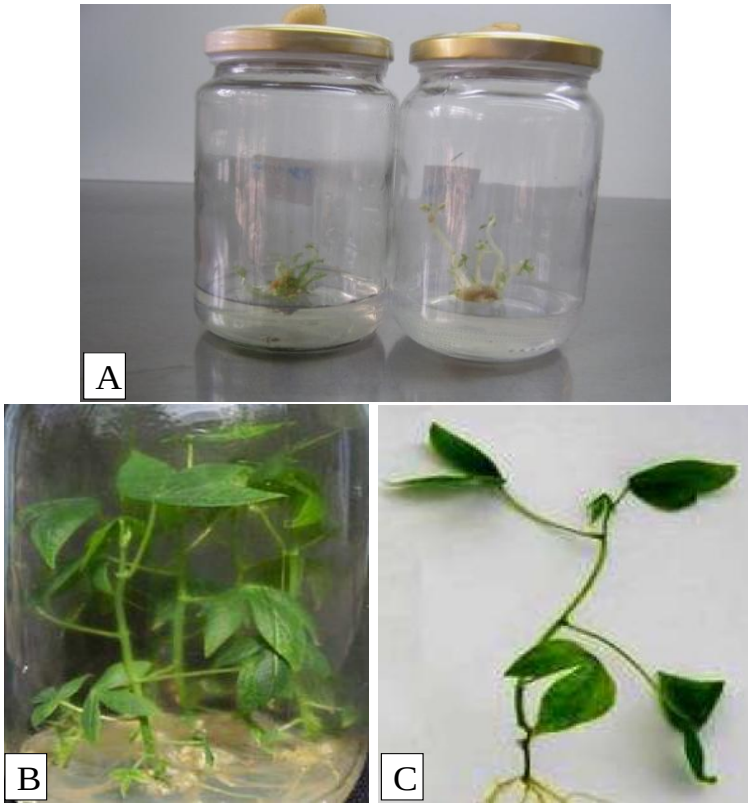

Figure 1. Morphological variations between the two cassava clones: *in vitro* proliferated shoots of clone 44/72-NR (right) and 44/72-NW (left) in 0.75 mg/L BAP and Kin media after two weeks of culture. (A), *In vitro* derived shoots of clone 44/72-NR (B) and 44/72-NW (C) in 0.75 mg/L BAP and Kin media after six weeks of culture.

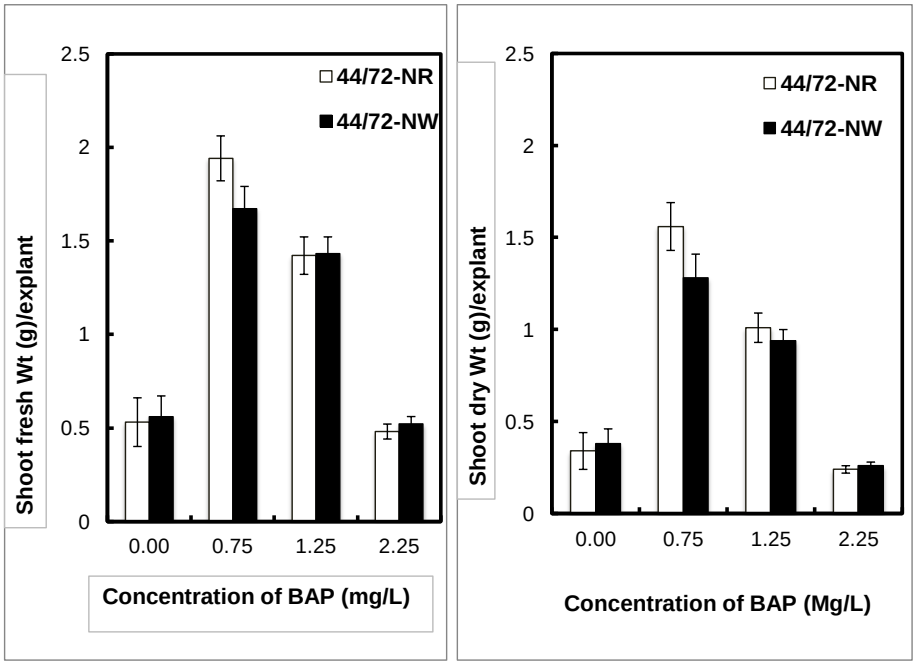

Figure 2. Interaction effect of BAP and clone on fresh and dry weights of cassava shoots after six weeks of *in vitro* culture.

Table 4. Mean square estimates of *in vitro* induced root parameters of cassava explants as affected by different concentrations and combinations of NAA, BAP and clone (ANOVA).

Treatment	DF	Root parameters/explant				Root : shoot weight ratio
		Number (No)	Length (cm)	Fresh weight (g)	Dry weight (g)	
NAA	3	1.210**	4.561**	2.997**	1.713**	0.259**
BAP	3	2.718**	3.794**	3.156**	2.507**	5.041**
NAA x BAP	9	0.141**	0.473**	0.555**	0.508**	0.102**
Clone	1	0.226**	0.299**	0.040	0.105**	0.042
NAA x Clone	3	0.001	0.029*	0.024	0.003	0.004
BAP x Clone	3	0.012	0.015	0.015	0.012	0.016
NAA x BAP x Clone	9	0.012	0.020**	0.019	0.013	0.013
R^2 (%)		91.2	96.7	92.1	92.1	78.0

*, ** indicate that Mean square values of root parameters considered are significant at 0.05 or 0.01 probability level, respectively.

Table 5. Interaction effect of NAA and Kin on in vitro root induction of cassava shoots after six weeks of culture.

Auxin source	Cytokinin source	Root growth parameters/explant			
NAA (mg/L)	Kin (mg/L)	Number (No)	Fresh weight (g)	Dry weight (g)	Root : shoot weight Ratio
0.0	0.0	3.50^{d}	3.21^{c}	2.05^{b}	2.50^{c}
	2.5	2.48^{g}	3.19^{c}	1.91^{c}	2.17^{d}
	5.0	2.10^{h}	1.83^{f}	1.09^{f}	0.86^{g}
	7.5	1.58^{k}	1.51^{g}	0.86^{g}	0.19^{i}
0.5	0.0	6.14^{a}	7.83^{a}	5.95^{a}	3.16^{a}
	2.5	3.86^{c}	3.57^{b}	2.08^{b}	2.56^{c}
	5.0	2.74^{f}	2.31^{e}	1.31^{e}	1.06^{fg}
	7.5	1.97^{i}	1.46^{gh}	0.81^{g}	0.54^{h}
1.0	0.0	4.13^{b}	2.55^{d}	1.82^{c}	2.83^{b}
	2.5	3.15^{e}	2.22^{e}	1.46^{d}	3.04^{ab}
	5.0	2.18^{h}	1.43^{gh}	0.90^{g}	1.19^{f}
	7.5	1.67^{jk}	1.17^{i}	0.71^{gh}	0.17^{i}
1.5	0.0	2.50^{g}	1.59^{g}	1.07^{f}	1.59^{e}
	2.5	2.25^{h}	1.29^{hi}	0.81^{g}	2.32^{cd}
	5.0	1.78^{j}	0.98^{j}	0.58^{hi}	1.20^{f}
	7.5	1.58^{k}	0.85^{j}	0.47^{i}	0.25^{i}
CV (%)		5.27	6.60	7.03	12.32

Means followed by the same letter within a column are not significantly different at 5% probability level, according to Student-Newman-Keuls multiple-range test (SNK).

weight (1.57 g) per explant as compared with clone 44/72-NW (Table 6) even when the *in vitro* rooting environment was the same for both. This might be due to the genetic variation that exists between the two clones. It could also be associated with long and high number of main and secondary roots (Supplementary Data) of clone 44/72-NR when compared with that of clone 44/72-NW, which directly increase number of main root and root dry weight. Furthermore, this clone was produced higher number of leaves in the shooting phase (Figure 1), which might have helped in supplying higher amount of photosynthates to the root which is a strong sink. Corroborate with this finding, Smith et al. (1986) and Acedo (2009) in cassava, Anura (2009) and Geleta (2009) in sweet potato previously reported the responses of root number and dry weight as it was different for different cassava clones *in vitro*.

Acclimatization of *in vitro* derived plantlets

The ultimate success of *in vitro* propagation lies in the

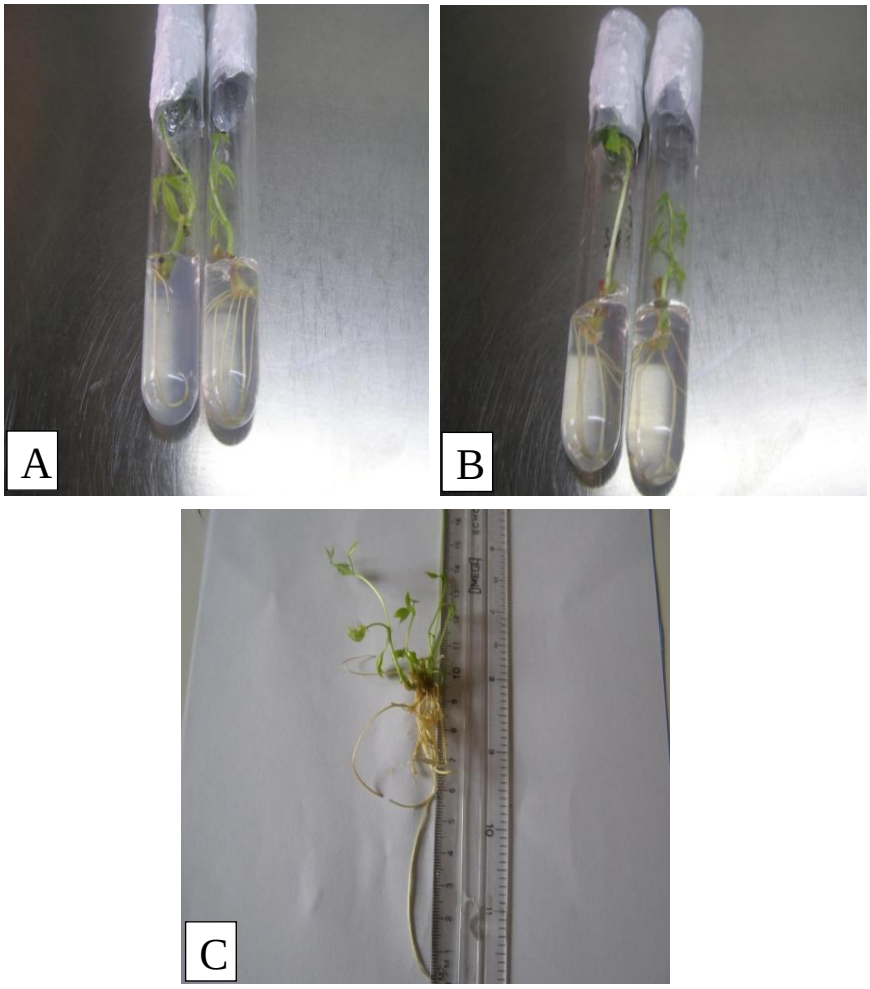

Figure 3. *In vitro* root development of clone 44/72- NR (A) and 44/72- NW (B) in 0.5 mg/L NAA media after four weeks of culture. Root length = 5.5 cm (C).

Table 6. Variation in number and dry weight of roots between two cassava clones after six weeks of *in vitro* culture

Clone	Parameters per explant	
	Number of roots (No)	Dry weight of root (g)
44/72-NR	2.86[a]	1.57[a]
44/72-NW	2.59[b]	1.42[b]

Means followed by the same letter within a column are not significantly different at 5% probability level, according to Student-Newman-Keuls multiple-range test (SNK)

successful establishment of plantlets in the soil. In this study, the *in vitro* rooted plantlets of both clones were planted onto a commonly recommended pre-sterilized potting mix of forest soil: well decomposed coffee husk: red sand (1:1:2 ratio by volume) to evaluate their survival rate under open field condition. Out of a total of 50 plantlets (25 plantlets from each clone) taken for final acclimatization, 93.3% of 44/72-NR and 86.7% of 44/72-NW survived and all plants were successfully transferred to field (Figure 4).

Conclusion

From the study, it was observed that *in vitro* shoot growth and development using nodal bud explants of the two cassava clones were best attained when the *in vitro* initiated plantlets were inoculated onto full strength solid MS medium supplemented with 0.75 mg/L BAP in combination with the same level of Kin. Likewise, the best *in vitro* root induction in the two cassava clones was observed when the *in vitro* derived shoots of both clone

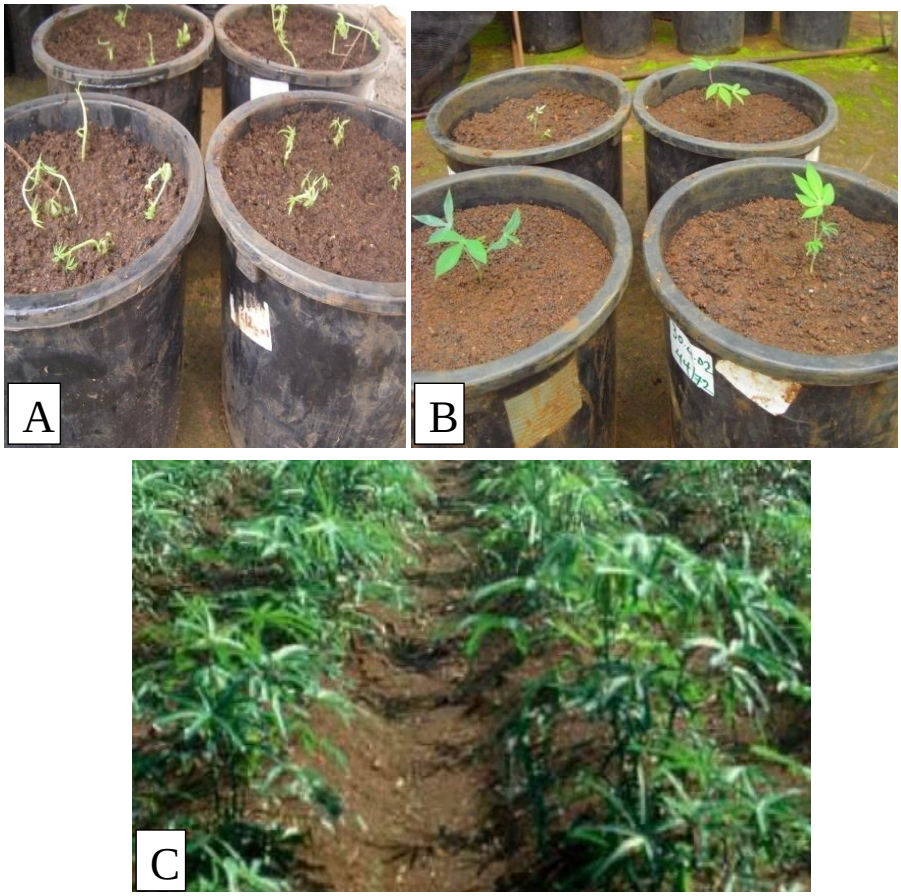

Figure 4. *Ex vitro* acclimatization of *in vitro* raised cassava plantlets: initiated plantlets during first day of acclimatization (A), seedlings after two weeks (B), survived seedlings under open field condition after 60 days (C).

were cultured in MS medium containing only 0.5 mg/L NAA. Thus, from our study, it can be concluded that for effective micro-propagation of the two cassava clones, MS medium need to be standardized in such a way that the shoot multiplication medium could be supplemented with a combination of BAP and Kin (each at 0.75 mg/L) and 0.5 mg/L NAA for root induction.

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Full Length Research Paper

Involvement of a hypersensitive-like reaction in tolerance to fire blight in pear (*Pyrus communis* L.)

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Infection by *Erwinia amylovora,* the causal agent of fire blight (FB) disease, induces in apple and pear host plants, the generation of reactive oxygen species (ROS). We analyzed at molecular level, the link between ROS production and susceptibility to bacterial infection. Gene expression time course showed that expression of chloroplast, mitochondrial and nuclear genes, whose transcription is redox-dependent, was down regulated or suppressed in tolerant cultivar, Harrow Sweet, in comparison with susceptible cultivar, Williams. Monitoring of oxidative burst by localization of hydrogen peroxide showed that oxidative burst was triggered faster in tolerant cultivar in response to infection. These results suggest that transcription of some redox-dependent genes of cytoplasmic organelles and nucleus, in the two cultivars, is regulated faster in the tolerant cultivar than in the susceptible one.

Key words: Chloroplast, electron transport Chain, *Erwinia amylovora*, mitochondria, *Pyrus communis*, redox.

INTRODUCTION

Erwinia amylovora in both compatible and incompatible interactions secretes three types of effector proteins: HrpN, HrpN/W and DspA/E (Bogdanove et al., 1998; Kim and Beer, 1998; Wei et al., 1992). HrpN inhibits electron transport chain (ETC) of mitochondria and triggers an oxidative stress in its incompatible interaction with tobacco cells (Xie and Chen, 2000). Venisse et al. (2001, 2002) also demonstrated the role of oxidative stress in compatible interactions of *E. amylovora* with susceptible hosts. Moreover, plant cells have a second ETC in their

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Abbreviations: DAB, 3,3´-Diaminobenzidine; **DEPC,** diethyl pyrocarbonate; **ETC,** electron transport chain; **FB,** fire blight; **Fd,** ferredoxin; **DspA/E,** disease specific type III effector protein; **GSSG,** glutathione disulfide; **Hrp,** harpin protein N and W; **PCD,** programmed cell death; **PEP,** plastid encoded RNA polymerase-PEP enzyme; **PQ,** plastoquinone; **PSI,** photosystem I; **PSII,** photosystem II; **PTK,** plastid transcription kinase; **ROS,** reactive oxygen species; **SLFs,** sigma-like transcription factors.

chloroplasts that in many aspects are similar to the ETC of mitochondria. There is biochemical evidence indicating the role of chloroplast ETC in oxidative burst of plant-pathogen interactions (Abdollahi and Ghahremani, 2011), and likewise Samuilov et al. (2002) proved that mitochondria and chloroplast are required in programmed cell death (PCD) of guard cells in pea leaves. They showed that illumination stimulated the CN-induced destruction of guard cells that contain both mitochondria and chloroplasts, but not of epidermal cells that contain mitochondria only.

Transcription in of cytoplasmic organelle genomes is dominantly under control of oxidative-reductive (redox) state. The role of redox state of chloroplasts on transcription of chloroplast and nuclear photosynthetic genes are well documented (Danon, 2002; Oswald et al., 2001; Pfannschmidt et al., 2001; Surpin et al., 2002). Trebitsh et al. (2000) showed a role for chloroplast thioredoxin in redox signaling and light-regulated translation of the *psbA* gene through disulfide bridge of RNA-binding complex and binding to 5´ region of *psbA* mRNA. Recent studies have identified two distinct RNA polymerases in chloroplasts; one out of two is an eubacteria-like multi-subunit type, with core components encoded by chloroplast genes (plastid encoded RNA polymerase-PEP enzyme) (Maliga, 1998). The major PEP core is surrounded by sigma-like transcription factors (SLFs). SLFs properties can be reversibly altered by phospho/dephosphorylation of a kinase (Baginsky et al., 1999), named plastid transcription kinase (PTK) (Baginsky et al., 1997). PTK phosphorylation of SLFs that is under control of redox state of chloroplasts leads to decrease transcription of chloroplast genes (Baginsky et al., 1999). In addition, there is evidence indicating that redox controls the transcription rate in the genome of mitochondria through activity of DNA-topoisomerase I (Konstantinov et al., 2001). Konstantinov and Tarasenko (1999) showed that redox poise of mitochondria controls the activity of DNA-topoisomerase I via the reduction/oxidation of a critical disulfide bridge. Venisse et al. (2001, 2002) demonstrated that *E. amylovora* invades host tissues by production of reactive oxygen species (ROS). Oxidative stress leads to a more oxidizing cell redox potential and consequently switches on or turns off some pathways, or transcription and translation of genetic information. Plant cells can distinguish different degrees of oxidant exposure (Cooper et al., 2002), and intensity of oxidative stress can control molecular events in the host cells.

To date, however, the biochemical evidences are not supported by molecular evidences on expression of mitocondrial and chloroplast genes whose regulation is redox-dependent, and nuclear genes encoding for chlorophyll synthesis, therefore still remain unclear whether the biochemical differences observed between tolerant and susceptible pear cultivars are regulated at gene expression level.

The aim of this study was to compare specific molecular events in a susceptible and a tolerant pear cultivar in responses to infection by *E. amylovora*. Our results provide useful hints to analyze the mechanisms leading to susceptibility and resistance in pears to fire blight, and develop more appropriate strategies to reduce damage caused by activation of host defense mechanisms.

MATERIALS AND METHODS

Bacterial strain, plant materials and infection

E. amylovora Ea273 was obtained from American Type Culture Collection (ATCC No. 49946). Pathogenicity of this bacterium was tested by inoculating *in vitro* shoots of Williams cultivar, through surgical removal of apices by a sterile scalpel, previously soaked in the bacterial suspension. Optimum concentration of inoculum and *in vitro* propagation of plant materials have been previously described (Abdollahi et al., 2004).

Five to six cm long *in vitro* grown shoots of two pear cultivars, Williams (susceptible) and Harrow Sweet (tolerant), were inoculated by adding 100 µl of a bacterial suspension (OD = 2 at A_{600}, in phosphate buffer pH = 7) to the surface of growth medium in 10 replications. All shoots were transferred to 16 h light photoperiod at 23 ± 1°C. Evolution of necrosis was compared in dark and constant light conditions (using cool white florescent lamps at 40 µmol m^{-2} s^{-1} photon flux) after basal inoculation of the shoots.

Detection of H_2O_2 by DAB staining method

H_2O_2 generation in the inoculated and control shoots of each cultivar was localized, using 3,3´-diaminobenzidine (DAB)-HCl (Sigma-Aldrich, Italy) as described by Thordal-Christensen et al. (1997). The shoot axes was catted in in 5-mm-long pieces, in three replications, which were soaked in an acidic water solution (pH = 3.8) containing 1 mg/ml DAB and 0.1% (v/v) Triton and incubated at 30°C (120 rpm) for 6 h. Samples were catted lengthwise and H_2O_2 traces were detected under stereomicroscope by red, purple and brown colors of the stem tissues. Data were expressed in percentage of produced H_2O_2.

Plant DNA and mRNA extraction

One-week-etiolated shoots of plant materials were used for DNA extraction in three replications by modified Sul and Korban (1996) method and quantified at A_{260}. Plant mRNAs were extracted from 0.1 g of plant materials in three replications by QuickPrepTM Micro mRNA purification kit (Amersham Pharmacia Biotech, Italy). This was done after the acidification of the medium indicated adequate activity of bacteria but before the appearance of necrotic symptoms of disease (Figure 1A). mRNAs were extracted from plant materials at least 3 h after beginning the light photoperiod, and dissolved in 10 µl diethyl pyrocarbonate (DEPC) treated water and quantified at A_{260}.

cDNA were synthesized, using 115 M-MuLV Reverse Transcriptase (Amersham Pharmacia Biotech, Italy). Each cDNA synthesis reaction was performed by using 100 ng mRNA, 0.5 µg of pd(T)12-18 as primer for the first strand cDNA synthesis in a total volume of 50 µl. cDNA synthesis was carried out at 42°C for 30 min, followed by deactivation of M-MuLV Reverse Transcriptase at 95°C for 5 min. Absence of DNA in the mRNA solutions was verified by following the expression of *eIF1*-α housekeeping gene (elongation factor) that on its genomic tested fragment has a 100 bp intron. All PCR reactions were conducted in the presence of 10 ng cDNA or 100 ng DNA, 1 µM of each forward and reverse primers (Table 1),

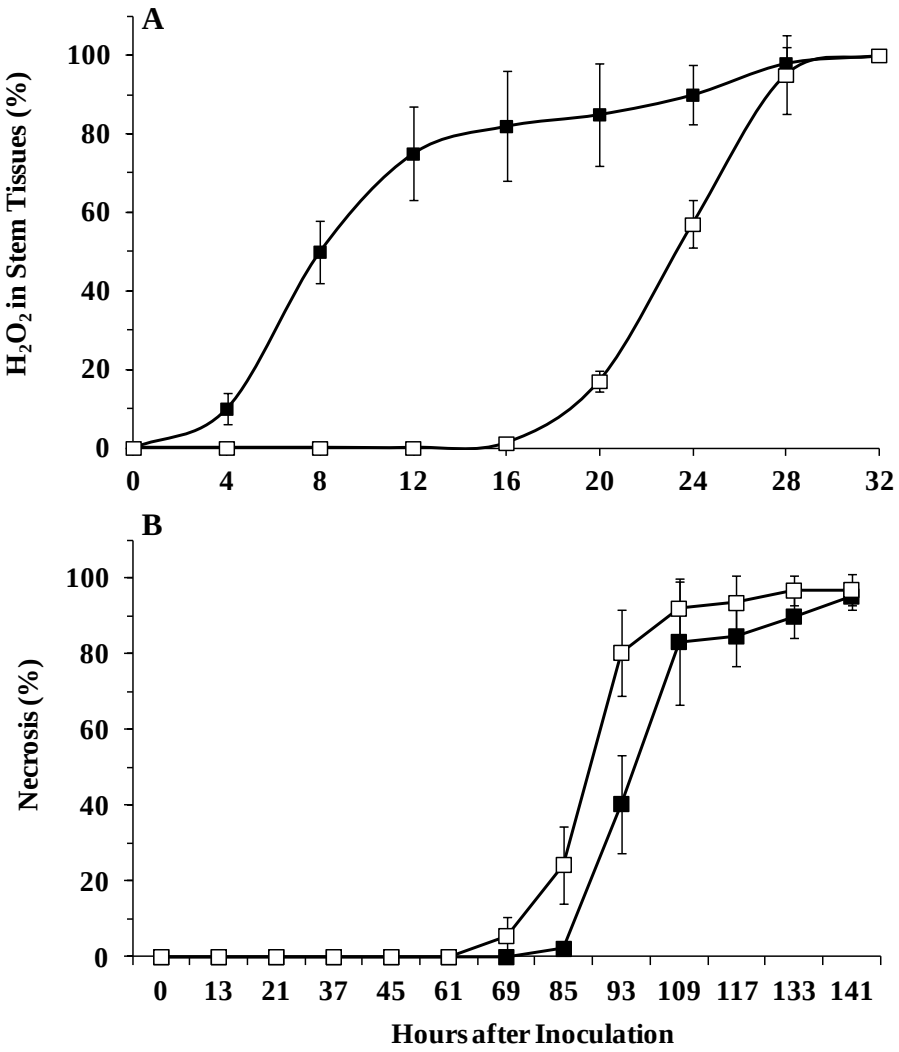

Figure 1. Progress of necrosis in the basal inoculated shoots to determine appropriate time for mRNA extraction (A), and localization of H_2O_2 generation in the stem tissues of the basal inoculated shoots of pear cvs. Williams (susceptible) and Harrow Sweet (tolerant) triggered by attack of *E. amylovora* (B).

0.75 mM $MgCl_2$, 2.5 µM dNTP, 1X PCR buffer and 1 unit Taq-DNA polymerase (Amersham Pharmacia Biotech, Italy). The PCR products were separated and visualized on 1% (w/v) agarose-ethidium bromide gel.

Designing of primers and DNA sequencing

DNA sequences of candidate genes were aligned by Multalin software (Corpet, 1988) and the primers were designed on the most conserved regions of the sequences. Partial sequences of chloroplast genes *psbA*, *psbB*, *psbC* and *psbD* (on DNA) and mitochondrial gene *nad4* (on cDNA) were sequenced to verify their identity and deposited in Gene Bank (Table 1).

Expression of candidate nuclear, chloroplast and mitochondrial genes

The gene expression studies were done in two gene groups, the housekeeping and redox-dependent-transcription genes (Table 1). Expressions of genes were studied at least in two replications, through PCR-amplification, by using cDNA as templates. PCR profiles were adjusted by using genomic DNA to obtain a single amplicon with expected length, except the *actin* gene.

Topology prediction of effector proteins of *E. amylovora*

The amino acid sequences of the effector proteins of *E. amylovora* (Accession of *hrpN*, *hrpW* and *dspA/E*, Q01099, AAF63402 and AAC62315, respectively) were obtained from NCBI database. Putative secondary structures of HrpN, HrpW and DspA/E proteins were predicted by GOR3 (Gibrat et al., 1987), GOR4 (Garnier et al., 1996), HNN (Guermeur, 1997), SOPM (Geourjon and Deléage, 1994) and SOPMA (Geourjon and Deléage, 1995) Internet software. Putative transmembrane α-helices in these proteins were predicted, using TMHMM (Moller et al., 2001), DAS (Cserzo et al., 1997), HMMTOP (Tusnady and Simon, 1998) and TMpred (Hofmann and Stoffel, 1993) programs.

Table 1. Sequences of primer used for amplification of genomic DNA and study of expression of different chloroplastic, mitochondrial and nuclear genes in the interaction between pear genotypes and *E. amylovora.*

	Gene	Gene product	Bases from start codon[a]	Primer Forward	Fragment length (mRNA)	Primer reverse	Ta (°C)
Chloroplast	*psbA*	D1 in PSII	243	5'-GAAAACCGTCTTTACATTGG-3'	942	5'-GTTGTGAGGATTACGTTCAT-3'	48
	psbB	PS II 47 kDa protein	9	5'-CCTTGGTATCGTGTTCATAC-3'	601	5'-CAATATGATGAGARGCTGTTC-3'	48
	psbC	PS II 44 kDa protein	3	5'-GAAACGCTCTTTAATGGAAC-3'	641	5'-ACACTAACAATCCAHCCTTC-3'	48
	psbD	D2 in PSII	300	5'-GATTTTACTCGTTGGTGTCA-3'	752	5'-GTTTCCACGKGGTARAACCTC-3'	48
	23S rRNA	23S ribosomal RNA	410	5'-TGTGAATCAGCAAGGACCAC-3'	386	5'-TAACCACAACTCATCCGCTG-3'	55
Mitochondria	*atp1*	ATPase subunit 1	728	5'-TCCGCGATAATGGAATGCAC-3'	384	5'-AAGCCGACGTTAATAGCAGG-3'	55
	nad1	NADH dehydrogenase subunit 1	37	5'-CACTTCTACTAGGAGTAGCC-3'	266	5'-CGGATCTGACAATACCATACC-3'	55
	nad3	NADH dehydrogenase subunit 2	88	5'-CCAATAGTTCGACCTATCCAG-3'	207	5'-CATAGAGAGATCCAATCGTC-3'	55
	nad4	NADH dehydrogenase subunit 4	212	5'-CCTTCGATGGCTTCCTTATG-3'	324	5'-CTGATATGCTGCCTTGATCT-3'	55
	26S rRNA	26S ribosomal RNA	1305-2291	5'-AACCATGTCGAAGGAACTCG-3'	301	5'-TTACACCATTCGTGCAGGTC-3'	55
Nucleus	*elF1-α*	Elongation factor 1	608	5'-ATTGTGGTCATTGGYCAYGT-3'	707	5'-CCAATCTTGTAYACATCCTG-3'	55
	act	actin	163	5'-ACNGGNATGGTNAAGGCTGG-3'	594	5'-GTCNCKNACAATTTCCCGCTC-3'	55
	cab	Chlorophyll a/b binding protein	0	5'-ATGGCTDCYKCHACWATGGC-3'	659	5'-CCATTCTTRAKCTCCTTYACC-3'	48
	18S rRNA	18S ribosomal RNA	2641-2654	5'-GTGCTCAAAGCAAGCCTACG -3'	760	5'-CGATCAGATACCGTCCTAGT -3'	55

[a] mRNA shows the housekeeping genes in nucleus, mitochondria and chloroplasts.

Cytoplasmic organelles targeting analysis of *E. amylovora* effector proteins

Two programs, Predotar (Somanchi and Mayfield, 1999) and TargetP (Emanuelsson et al., 2000) were used to predict the existence of cytoplasmic and mitochondrial presequences in effector proteins of *E. amylovora*. In addition, all known presequences of mitochondrial protein import presequences, listed by Whelan and Glaser (1997), were aligned manually and their homologies were compared with effector proteins of *E. amylovora* by Multalin Internet software (Corpet, 1988). The amphipathicity of putative α-helices were predicted by PepTool software.

RESULTS and DISCUSSION

Hydrogen peroxide accumulation in infected shoots

Monitoring of oxidative burst, by histochemical detection of hydrogen peroxide using DAB-staining, indicated that infection induced an increase in H_2O_2 in the shoots of both cultivars, although time-course profiles were quite different (Figure 1A). In Harrow Sweet cultivar, cells responded with a rapid generation of H_2O_2 4 h after inoculation and H_2O_2 level reached the highest value after 28 h. In contrast, in Williams shoots the production of H_2O_2 was delayed up to 15 h and extended over the entire length of the shoots 28 h after inoculation.

It is worth noting that in both cultivars, evident symptoms of necrosis appear several hours after the H_2O_2 level reached the highest value, but the time of onset of these symptoms was cultivar dependent and was delayed in Harrow Sweet cultivar (Figure 1B).

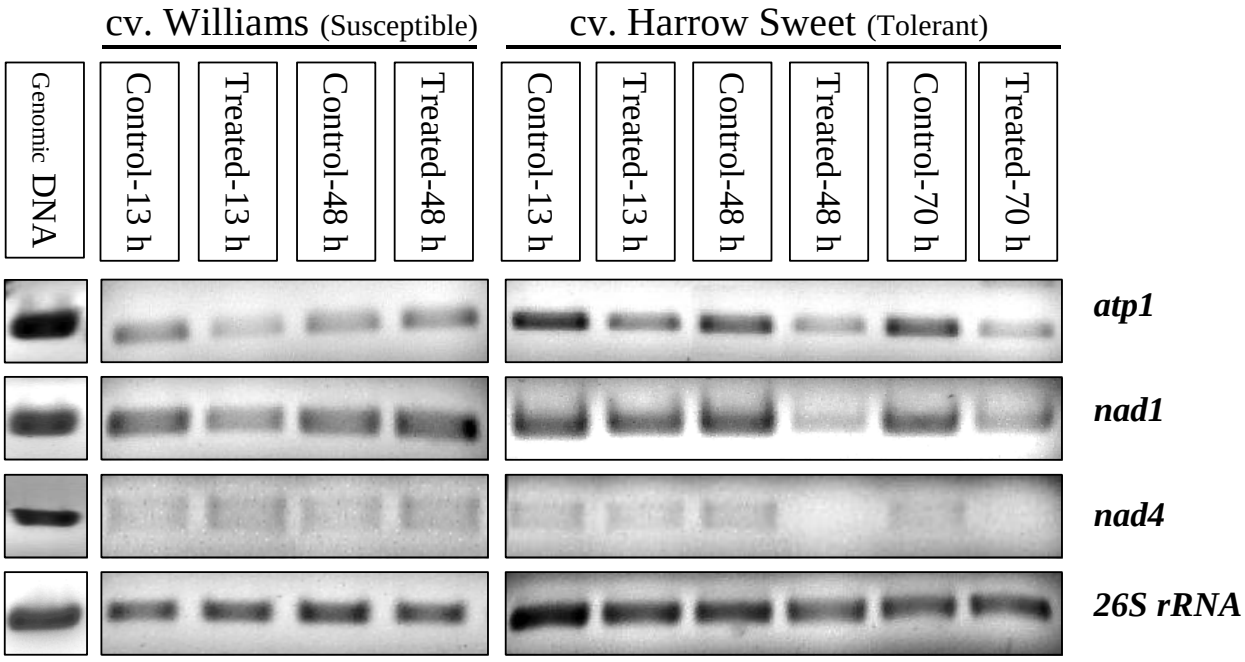

Figure 2. Expression of different mitochondrial genes, in control and inoculated shoots, of pear cvs. Harrow Sweet and Williams.

As reported in the literature, H_2O_2 can generate a redox poise in the organelles and affects the expression of redox-depending genes in chloroplast and mitochondria (Konstantinov and Tarasenko, 1999). We intended to test whether ROS production stimulated by *E. amylovora* infection affects the gene regulation of some organelles and nuclear genes.

Expression of mitochondrial and chloroplast genes in pear cultivars

In Harrow Sweet inoculated shoots, expression of mitochondrial *atp1* and *nad1* genes was gradually reduced while expression of *nad4* gene was completely suppressed after 48 h (Figure 2). In contrast, in Williams shoots no difference was observed in the expression of tested genes in both inoculated and control shoots (Figure 2). Transcription analysis of chloroplast genes (Figure 3) indicated that expression of *psbA* gene was strictly linked to the capacity of cultivars to tolerate the pathogen and early production of H_2O_2 (Figure 1); it resulted to dramatic recrease, 13 h after inoculation, in Harrow Sweet shoots, while it was slightly affected, 48 h after inoculation, in Williams shoots. No difference in the transcript level of *psbB*, *psbC* and *psbD* genes was detected in inoculated and control shoots of both cultivars (Figure 3).

It has been shown that a discrete number of ESTs involved in redox system and photosynthesis were modulated in responses to *E. amylovora* invasion (Sarowar et al., 2011; Baldo et al., 2010). Vrancken et al. (2013) suggested that rapid increase of ROS is one of the earliest plant responses to *E. amylovora* invasion. Observations made in inoculated shoots of Harrow Sweet cultivar where an early increasing of H_2O_2 production occurred few hours after inoculation, and down-regulation of *psbA* expression was detected 13 h after inoculation, indicates that a photo-production of H_2O_2 occur in the infected tissues. *psbA* gene encodes the protein D1 that plays a pivotal role in photosystem II (PSII). Light-sensing network and oxygen-evolving complex in PSII have been shown to be linked to plant defense against pathogen infection (Genoud et al., 2002; Abbink et al., 2002). The PSII plays an important role in preventing the accumulation of ROS (Krieger-Liszkay, 2005) therefore to activate the protective responses and to induce systemic acquired resistance by ROS are needed to down-regulate PSII activity (Fryer et al., 2003; Kulheim et al., 2002). Although in apple young leaves, photosynthetic activity was inhibited and chlorophyll florescence was changed by ferredoxin (Fd) dependent way, prior to the development of disease symptoms (Bonasera et al., 2006), however an up-regulation of some photosynthetic genes have been observed by Heyens and Valcke (2006) and Baldo et al. (2010), in apple invaded tissues. Bonasera et al. (2006) showed that pathogen effector DspE/A interacts with precursor-ferridoxin in the cytoplasm, thereby preventing its transfer into chloroplast, where it is usually converted to ferredoxin (Fd) and it serves as an electron carrier in photosystem I (PSI). Moreover, Singh et al. (2010) suggested that FIBRILLIN4, which is associated with photosystem II, could also play a part in fire blight infections, as the disease is more expressed in the knock-

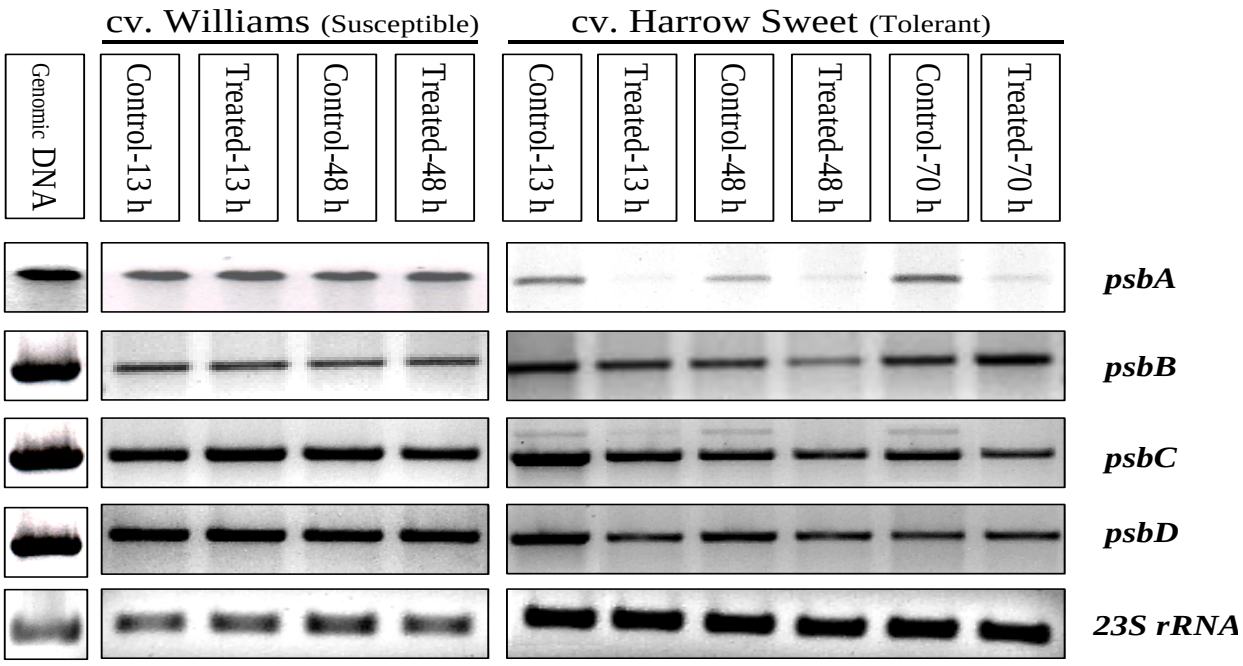

Figure 3. Expression of different chloroplast genes, in control and inoculated shoots, of pear cvs. Harrow Sweet and Williams.

down mutant. Oxidation and reduction of glutathione (GSH), described by Baginsky et al. (1999) can explain changes in *psbA* transcription by the effects of oxygen radicals, generated during infection by the pathogen. *In vivo*, most of the chloroplast glutathione is in the reduced GSH form that serves as a redox buffer and ROS scavenger. During oxidative stress of pathogen, the GSH oxidizes and forms glutathione disulfide (GSSG) and the chloroplast GSH:GSSG ratio decreases significantly, with a transient increase of GSSG and concomitant decrease in GSH redox state, resulting in decrease activity of PTK. Lower PTK activity leads to lower phosphorylation of SLFs in RNA-polymerase complex of chloroplasts and decreases its activity for transcription of genes such as *psbA*. In addition, expression of other photosynthetic genes, *psbC*, *psbB* and *psbD*, were not suppressed like the *psbA* gene, indicating different mechanism, which control the transcription and reduce the redox sensibility of these genes. These results are also in accordance with Pfannschmidt et al. (1999), which showed that the redox state of plastoquinone (PQ) effectively controls the redox poise of chloroplasts and transcription of the *psbA* gene, but transcription of other chloroplast genes were not affected by the PQ redox state.

It is known that an increase in photosynthetic activity usually induces the production of sugar and ATP that might prevent in *Malus* the colonization by *E. amylovora* by increasing host plant defense through the light sensing signaling pathway and by activation of additional defense related genes (Baldo et al., 2010). In our experiments, pear plantlets of both cultivars are grown in medium with a large amount of assimilate carbohydrate. This condition makes easier to highlight the differences existing between cultivars regarding fire blight susceptibility, because a suitable amount of energy source is available for both susceptible and tolerant cultivars shoots. In shoots of susceptible Williams cultivar exposed to light, necrotic symptoms of fire blight are delayed with respect to those exposed to darkness, thus supporting a role in the mechanisms of plant defense against pathogen infection of the light signaling pathways.

It has been reported that redox state of mitochondria controls transcription of mitochondrial genome by affecting the activity of DNA-topoisomerase I (Konstantinov et al., 2001). Therefore, it is possible that ROS generation, due to infection by the pathogen, alters the redox poise of mitochondria and changes the transcription rate of this organelle in the pear cells. Activity of DNA-topoisomerase I opens supercoiled mitochondrial DNA by introducing a transient single-strand break in the duplex and acts in a number of different DNA metabolisms, such as DNA replication, transcription and repair (Champoux, 2001). Therefore, it will be expected that any variation in redox poise of mitochondria and activity of DNA topoisomerase I, could alter the transcription of all mitochondrial genes similarly.

Our results showed that infection by the pathogen suppressed expression of some mitochondrial genes and concomitantly did not affect expression of *26S ribosomal-RNA* gene in the inoculated shoots of either cultivar. This indicates a role for at least a second mechanism controlling the transcription rate of mitochondrial genes. Oxygen

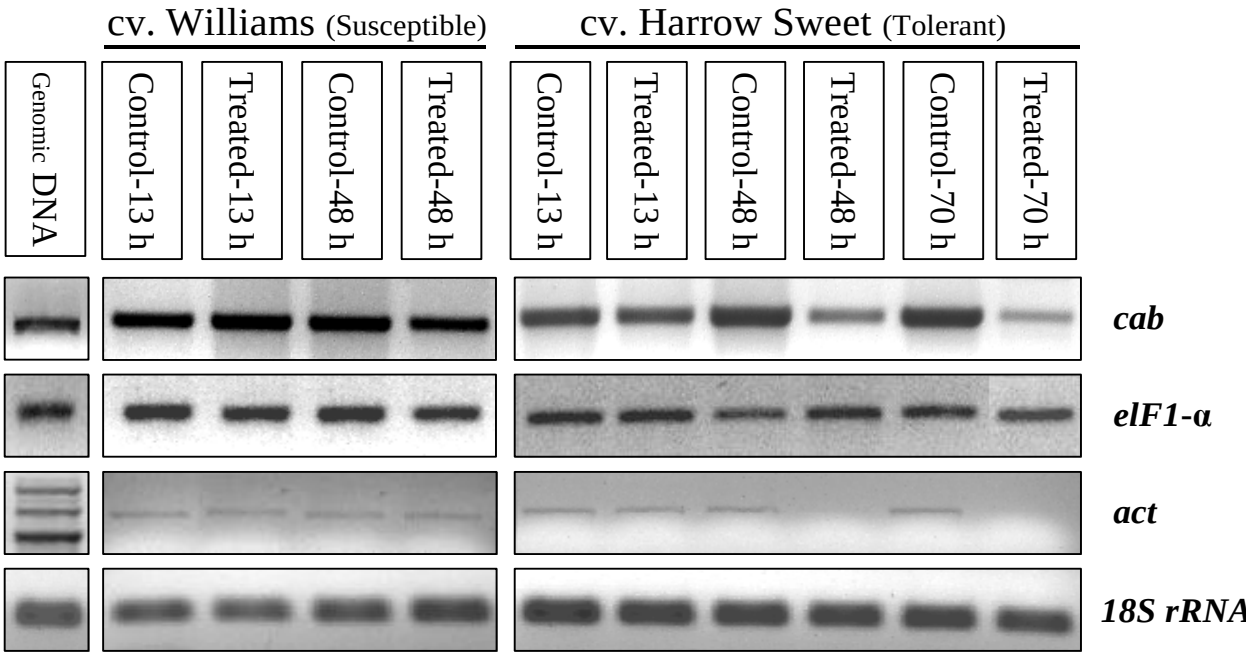

Figure 4. Expression of different nuclear genes in control and inoculated shoots of pear cvs. Harrow Sweet and Williams.

radicals generated in the mitochondria are very mobile and diffuse rapidly elsewhere in the cytoplasm and organelles (Moller, 2001). Subsequently, production of ROS by pathogen infection and penetration in the chloroplasts alters redox state and expression of some redox-dependent-transcription genes such as *psbA* in this organelle.

Expression of nuclear genes

Similar to the chloroplast genes, expression of nuclear genes varied with the genotypes and gene kind (Figure 4). Results showed that expression of nuclear genes of Williams cultivar were not affected by the infection, only the expression of the *cab* gene was slightly reduced at 48 h from inoculation. In Harrow Sweet cultivar, two housekeeping genes, *elF1*-α and *18S ribosomal-RNA*, expressed constantly up to 70 h in both control and inoculated shoots, while expression of *cab* and *act* genes showed a gradual reduction in the inoculated shoots (Figure 4). The constant expression of *elF*-α and *18S ribosomal-RNA* showed that even after 70 h after inoculation, RNA-polymerase of the nucleus had not been deactivated by pathogen infection.

Recent findings have confirmed the role of chloroplast signaling on transcription of photosynthetic nuclear genes (Somanchi and Mayfield, 1999). Several factors have been proposed as signaling intermediates, like tetrapyrrole, sugars and redox state of chloroplasts (Surpin et al., 2002). Oswald et al. (2001) demonstrated that transcription of nuclear photosynthetic *rbcS* and *cab* genes are under control of redox state of thioredoxin or glutathione system in plant chloroplasts, although the nature of this signaling is still unknown. In our work, the reduction of *cab* gene in the inoculated shoots could be a consequence of the chloroplast signaling to the nucleolus through changes in the redox poise of thioredoxin or GSH system of chloroplasts by oxidative stress of pathogen.

Topology prediction of effector proteins of *E. amylovora*

Xie and Chen (2000) showed that the effector proteins of *E. amylovora* interact with tobacco cells affecting the complex III and/or IV of mitochondrial ETC. However, the mechanism allowing these proteins to pass through mitochondrial membranes are not known, yet. Most of the proteins located in the chloroplasts and in the mitochondria are encoded by nuclear genes and synthesised in the cytoplasm. They are recognized through the signal sequences present in the N-terminal region of protein and transported through mechanisms of protein trafficking into the appropriate organelles by participation of chaperones (Glaser et al., 1998). In addition, mitochondria and chloroplasts are believed to have evolved from prokaryotic ancestors, and they still exhibit some functional similarities to the bacteria. For instance, at least two out of four protein transport systems in the chloroplasts and one system in the mitochondria are homologous to the translocation system in the inner membrane of bacteria (Moore et al., 1994; Schnell, 1998). It is possible that *E. amylovora* produces effector proteins that target the mitochondria through these protein trafficking systems and affecting ETC of this organelle. Secondary structure analysis of the effector proteins of *E. amylovora* individuate

Table 2. Targeting prediction of *E. amylovora* effector proteins to the plant mitochondria and chloroplasts by Predotar (Small 2003) and TargetP (Emanuelsson et al., 2000) software.

Effector protein	Predotar			TargetP						Predicted location
	Cp score[a]	Mit score[b]	Location	cTP[c]	mTP[d]	SP[e]	Other[f]	Location	RC[g]	
HrpN	0.012	0.133	Neither	0.210	0.134	0.026	0.522	---	4	Neither
HrpW	0.029	0.062	Neither	0.469	0.094	0.040	0.465	C	5	Chloroplast
DspA/E	0.065	0.124	Neither	0.133	0.155	0.018	0.727	---	3	Neither

[a, c]Chloroplasts targeting probability; [b,d]mitochondria targeting probability; [e]secretory pathway targeting probability; [f]any other location targeting probability; [g]reliability class (RC=1 as the highest reliability).

Table 3. Evolution of fire blight necrosis in the basal inoculated shoots of pear cv. Williams under continuous dark and light conditions. Values represent the average of two independent experiments ± 2SE.

Hours after inoculation	Light	Dark
0	0.0	0.0
12	0.0	0.0
20	0.0	0.0
32	0.0	0.0
40	0.0	0.0
52	0.6 ± 0.5	2.5 ± 2.1
60	1.3 ± 1.0	6.3 ± 5.3
72	8.1 ± 5.1	15.0 ± 4.4
80	25.0 ± 4.5	64.0 ± 5.4
92	75.0 ± 4.4	90.0 ± 6.5
100	90.0 ± 4.2	100.0 ± 0.0
108	100.0 ± 0.0	100.0 ±0.0

9, 3 and 7 putative α-helices, in HrpN, HrpW and DspA/E proteins, respectively, as predicted by five different software (see materials and methods). However, only in the HrpN protein a high significant and consistent putative transmembrane helix motive, about 20 amino acids in size, is present. HrpN is a potential effector candidate to elicit either a hypersensitive reaction on non-host plants or a pathogenic reaction on host plants. Therefore, the presence of transmembrane alpha helix motive may explain the role of HrpN in pathogenicity of *E. amylovora*.

Prediction of HrpN, HrpW and DspA/E targeting the chloroplasts and mitochondria by Predotar software did not identify any signal sequences in the N terminal region (Table 2). However, the analyses run using TargetP software predicted a possible HrpW chloroplast-target signal sequence. Since it has been shown that the chloroplasts are involved in PCD of guard cells in pea leaves (Samuilov et al., 2002), it may be postulated that the effector proteins of pathogen interact with photosystem II, probably by down regulation of *psbA* gene, triggering an oxidative stress of cells of infected plants. If this hypothesis is true, we should expect a slower progress of the necrosis in plants exposed to light than those exposed to dark condition.

Delay of the progress of necrosis under light conditions

Comparison between the evolution of necrosis in the inoculated shoots of Williams cultivar in the dark and in the light showed that necrosis progressed faster in the dark (Table 3). This difference could not be caused by the inhibitory effects of light on pathogen, but in the absence of flux of electrons in the chloroplast ETC can be due to the reduced capacity of plant to contrast the pathogen. This event can be explained by the interaction between HrpW and ETC of chloroplasts, which generates oxidative stress. It means that under light condition, HrpW interacts with chloroplast ETC, supporting mitochondria as a second source of ROS generation and decreasing the invasion of pathogen. Experiments to unravel the role of HrpW and chloroplast on the progress of fire blight are in progress.

Conclusion

With this study, the relationship between the two events are shown for the first time, but further researches need to be carried out to identify the biological determinants that generate the differences between tolerant Harrow Sweet and susceptible Williams cultivars. In fact, the causal relationship between ROS activity and down-regulation of expression of redox-dependent nuclear, mitochondrial and chloroplast genes is not straightforward, because plant resistance in general, and induced resistance in particular, is an emergent property of a plant that results from the combined action of multiple genetic, biochemical, physiological and morphological traits that interact with one another and that are expressed heterogeneously in space and time.

Conflict of Interests

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

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Full Length Research Paper

Influence of 24- epibrassinolide on *in vitro* shootlets regeneration *via* direct organogenesis of *Phaseolus vulgaris* L.

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This study aimed to optimize an *in vitro* shootlets regeneration system via direct organogenesis for *Phaseolus vulgaris* L. cv. Brunca using 24- epibrassinolide (24-Epi). The best medium for shootlets proliferation as well as growth parameters was MS supplemented with 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi. Stem explants recorded the best results of shootlets proliferation percentage compared with leaf or root explants, respectively. MS medium supplemented with 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi gave the best results of recovered shootlets (%); photosynthetic pigments, number of leaves, number of shootlets and shootlet length (cm). From the obtained results it can be recognized that, the behavior and trends of 24-Epi on *in vitro P. vulgaris* cultures media may be oriented and arranged to the behavior of auxins in the nutrient medium for roots formation and as a cytokinin in shootlets proliferation.

Key words: *Phaseolus vulgaris* L., 24- epibrassinolide, shootlets regeneration, direct organogenesis.

INTRODUCTION

Common bean (*Phaseolus vulgaris*) is one of several crop species belong to the Fabaceae family, commonly known as grain legumes or pulses. In total, there are about 650 genera and 18,000 species in the legume family (Hymowitz, 1990). Common bean is a very important source of vegetable protein, especially in those regions of the world in which animal and fish protein is scarce. Common bean satisfy 22% of the total protein requirement worldwide (Delgado-Sanchez et al., 2006) and account for over 50% of all legumes consumed globally (Blair et al., 2006). Like most grain legumes, common bean is rich in the essential amino acid lysine (Babaoglu et al., 2000; Popelka et al., 2004).

Plant biotechnology, together with conventional breeding methods, could facilitate bean improvement since resistance or tolerance to biotic and abiotic stress and could increase the seed quality, plant architecture and reproduction modes (Veltcheva et al., 2005). Nevertheless, a reliable and efficient *in vitro* culture system that results in efficient differentiation, shoot development and whole plant regeneration is an essential requirement for improvement of common bean through genetic transformation or mutagenesis (Varisai Mohamed et al., 2006).

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A general feature of common bean genotypes is their recalcitrance to regenerate *in vitro*. This is because they produce significant amounts of phenolic compounds *in vitro* which inhibit their regeneration. Regeneration of many legumes has been successfully obtained by means of somatic embryogenesis and organogenesis and has been reported by numerous researchers. However, the regeneration of common bean by somatic embryogenesis has been unsuccessful because of the recalcitrance of this species and the inability to advance the development of embryos past the globular stages. Similarly, organogenesis has been successful in very few occasions (Carvalho et al., 2000; Guidolin, 2003). This limits the possibilities of genetic transformation of common bean. In order to overcome this difficulty, it is necessary to analyze and determine the chemical conditions and the type of explant that allow a reliable regeneration of plants by both, somatic embryogenesis and organogenesis.

Brassinosteroids (BRs) are naturally occurring plant growth regulators, which exhibit structural similarities to animal steroid hormones (Mandava, 1988). BRs affect a variety of physiological processes, including stem elongation, pollen tube growth, leaf bending and epinasty, root inhibition, fruit development, ethylene biosynthesis, proton pump activity, xylem differentiation, photosynthesis, and gene expression (Dhaubhadel et al., 1999; 2002; Singh and Shono, 2005). Moreover, BRs can induce plant tolerance to a variety of biotic and abiotic stresses (Xia et al., 2009). BRs were also found to have an activity *in vitro*. They were reported to increase the rate of cell division and colony formation of Chinese cabbage mesophyll protoplasts (Nakajima et al., 1996) and *Petunia hybrida* protoplasts (Oh and Clouse, 1998). BRs were also found to be essential for the differentiation of isolated *Zinnia* mesophyll cells into tracheary elements (Iwasaki and Shibaoka, 1991) and in the morphogenesis of *Arabidopsis* (Li et al., 1996). Moreover, BRs promoted adventitious shoot regeneration from segments of cauliflower hypocotyls (Sasaki, 2002).

The main objectives of the present study are to investigate the efficiency of 24- epibrassinolide (24-Epi) on *in vitro* shootlets regeneration *via* direct organogenesis as well as study the interaction between 24-Epi and IAA on rootlets formation of *Phaseolus vulgaris* L. cv. Brunca.

MATERIALS AND METHODS

This study was carried out in Plant Biotechnology Department, Genetic Engineering Division, National Research Centre, Dokki, Cairo, Egypt and Botany Department, Faculty of Women for Arts, Science and Education, Ain Shams University, Cairo, Egypt.

Sterilization and *in vitro* germination

Seeds of *P. vulgaris* L. cv. Brunca were obtained from Faculty of Agriculture, Cairo University, Egypt. The process of sterilization and *in vitro* germination of *P. vulgaris* seeds was carried out according to the method described by Nafie et al. (2013).

Plant culture and media composition

Murashige and Skoog, 1962 salts medium (MS) including vitamins, glycine and supplemented with 30 g L^{-1} sucrose, and solidified with 7 g L^{-1} agar was used in this study. The pH of the pre-agar MS medium was adjusted to 5.8 using 0.1 M NaOH before autoclaving. Cultures were incubated in illumination light condition at 26 ± 1°C and sufficient fluorescent light of 1500 lux for 16/8 h (light/dark) photoperiod for shootlets regeneration and 1000 lux for rootlets formation after incubation in complete darkness for three days.

Growth regulators used in this study

24-Epibrassinolide (24-Epi) was first dissolved in dimethyl sulfoxide (DMSO) before treatment according to Shujie et al. (2013), 2,4-dichlorophenoxy acetic acid (2,4-D). 3-indole acetic acid (IAA), thidiazuron (TDZ) were purchased from Sigma company. IAA was filtered through 0.2 µm pore size in Laminar Air flow cabinet**.**

Shootlets regeneration

For achievement of direct shootlet regeneration, different explants excised from three weeks old *in vitro* growing seedlings were cut into convenient sizes using scalpel blade, aseptically 3-4 mm length from stem, root and 4 mm^2 size from leaf. Aseptically explants were cultured on MS medium supplemented with 24-Epi alone or in combination with thidiazuran (TDZ) as follow: S_0 = MS free growth regulators; S_1 = MS + 0.025 mg L^{-1} 24-Epi; S_2 = MS + 0.05 mg L^{-1} 24-Epi; S_3 = MS + 0.1 mg L^{-1} 24-Epi; S_4 = MS + 2 mg L^{-1} TDZ; S_5 = MS + 2 mg L^{-1} TDZ + 0.025 mg L^{-1} 24-Epi; S_6 = MS + 2 mg L^{-1} TDZ + 0.05 mg L^{-1} 24-Epi; S_7 = MS + 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi.

Each treatment consisted of 5 replicates (jars) and each replicate contained three explants. Cultures were maintained by sub-culturing using the same medium at three weeks intervals from incubation. After 4 weeks from incubation, frequency of recovered shootlets from direct organogenesis (%), photosynthetic pigments (mg g^{-1} FW**)**, shootlet length (cm), number of shootlets/explant, number of leaves and shootlets fresh/dry weights (g/jar) were recorded.

Roots formation

The healthy regenerated shootlets with 6-7 cm length which resulted from S7 medium were transferred to MS roots formation medium which supplemented with different concentrations of 24-Epi (0.025, 0.05 and 0.1 mg L^{-1}) alone or in combinations with 0.5 mg L^{-1} IAA as follow: R_0 = MS free growth regulators; R_1 = MS + 0.025 mg L^{-1} 24-Epi ; R_2 = MS + 0.05 mg L^{-1} 24-Epi; R_3 = MS + 0.1 mg L^{-1} 24-Epi; R_4 = MS + 0.5 mg L^{-1} IAA; R_5 = MS + 0.5 mg L^{-1} IAA + 0.025 mg L^{-1} 24-Epi ; R_6 = MS + 0.5 mg L^{-1} IAA + 0.05 mg L^{-1} 24-Epi; R_7 = MS + 0.5 mg L^{-1} IAA + 0.1 mg L^{-1} 24-Epi.

Each treatment consisted of 5 replicates (jars) and each replicate contained three shootlets. The cultures were incubated in a controlled growth chamber in complete darkness for 3 days at 26 ± 1°C then transferred to light condition (1000 Lux for 16/8 h light/dark). Frequency of shootlet producing root (%); number of roots; root length (cm) and roots dry weight (g/jar) were recorded after four weeks of cultivation.

Table 1. Effect of interaction between media composition and explant types on frequency of direct shootlets regeneration, number of shootlets/explant, number of leaves/shoot , shootlet length and shootlet fresh/ dry weights of *P. vulgaris* L. cv. Brunca.

Culture media composition	Shootlets (%)	Number of shootlets/explant	Number of leaves/shoot	Shootlet length (cm)	F.W (g/jar)	D.W (g/jar)
Leaf explants						
S_0 = MS free growth regulators	-	-	-	-	-	-
S_1= MS + 0.025 mg L^{-1} 24-Epi	-	-	-	-	-	-
S_2= MS + 0.05 mg L^{-1} 24-Epi	-	-	-	-	-	-
S_3= MS + 0.1 mg L^{-1} 24-Epi	-	-	-	-	-	-
S_4= MS + 2 mg L^{-1} TDZ	50^{cd}	6.5^{d}	12.5^{f}	6.2^{f}	0.607^{e}	0.02^{d}
S_5= MS + 2 mg L^{-1} TDZ + 0.025 mg L^{-1} 24-Epi	50^{cd}	7^{d}	16^{d}	6.5^{f}	0.944^{c}	0.03^{cd}
S_6= MS + 2 mg L^{-1} TDZ + 0.05 mg L^{-1} 24-Epi	83^{ab}	6.5^{d}	17^{cd}	7.3^{f}	1.109^{b}	0.05^{bc}
S_7= MS + 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi	83^{ab}	7^{d}	21^{b}	7.2^{bc}	1.249^{a}	0.05^{abc}
Stem explants						
S_0 = MS free growth regulators	-	-	-	-	-	-
S_1= MS + 0.025 mg L^{-1} 24-Epi	-	-	-	-	-	-
S_2= MS + 0.05 mg L^{-1} 24-Epi	-	-	-	-	-	-
S_3= MS + 0.1 mg L^{-1} 24-Epi	-	-	-	-	-	-
S_4= MS + 2 mg L^{-1} TDZ	67^{bc}	7^{d}	14.5^{e}	6.2^{g}	0.41^{f}	0.03^{d}
S_5= MS + 2 mg L^{-1} TDZ + 0.025 mg L^{-1} 24-Epi	67^{bc}	8^{c}	$17.^{cd}$	7^{ef}	0.743^{d}	0.06^{ab}
	83^{ab}	8.5^{bc}	17.5^{cd}	9^{ab}	0.929^{c}	0.06^{ab}
	90^{a}	12^{a}	23.5^{a}	9^{ab}	0.928^{c}	0.07^{a}

Means having the same letters in a column were not significantly different 0.05 level.

Photosynthetic pigments extraction and estimation

Photosynthetic pigments (chlorophyll a, b and carotenoids) were estimated according to the method of Lichtenthaler and Wellburn (1987). Definite weight from each treatment (0.2 g) were homogenized in acetone 85% (v/v), filtered and made up to a final volume of 4 ml. Pigments at each maximum absorption wave length were estimated using (JENWAY 6305 UV-VIS) Spectrophotometer apparatus and absorbance was recorded at 646 and 663 nm for chlorophyll assay and 470 nm for carotenoids. Calculated using the formula given below:

Chl *a* mg/g F.W = (12.25 A_{663} - 2.79 A_{646}) × V/W
Chl *b* mg/g F.W = (21.21 A_{646} - 5.1 A_{663}) × V/W
Car mg/g F.W = (1000 A_{470} - 1.8 Chl *a* - 85.02 Chl *b*)/198

Where, A = Absorbance at 663, 646 and 470 nm. V = Final volume of chlorophyll extract in 85% acetone (ml). W = Fresh weight of the leaf tissue (g).

Statistical analysis

The test of least significant difference (LSD) at the level of 0.05% significance was used to examine differences among treatment means and interactions. Data were statistically analyzed using MSTAT-C software package according to the method described by Freed et al. (1989).

RESULTS

Effect of media composition and type of explants on efficiency of shootlets regeneration

Data in Table 1 represent the effect of cultures media and type of explants on shootlets regeneration *via* organogenesis. Data clearly showed significant differences between treatments concerning the frequency of direct shootlets regeneration (%), number of shootlets, number of leaves, shootlet length (cm) and fresh/dry weights of *P. vulgaris* L. cv Brunca after 4 weeks of culturing on MS medium supplemented with 2 mg L^{-1} TDZ and different concentrations of 24-Epi. In this regard, MS medium supplemented with 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi significantly gave the best results of shootlets regeneration compared with other treatments. On the other hand, among the seven tested media; S_4, S_5, S_6 and S_7 only induced shootlets regeneration using leaf and stem explants compared with S_0 (free growth regulators), S_1, S_2 and S_3 which recorded no responses to shootlets regeneration. The obtained results indicated that stem explant produced the highest frequency of shootlets (90%) followed by leaf explant (83%) on MS medium

Figure 1. Regenerated shootlets from stem (A) and leaf (B) segment of *P. vulgaris* after the formation ofcallus around the explants on MS medium supplemented with 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi. (5×).

supplemented with 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi, respectively (Figure 1A, B). Furthermore, the obtained results clearly indicated that there were no responses to shootlet regeneration on MS medium supplemented with 24-Epi alone. Whereas, adding TDZ (2 mg L^{-1}) alone or in combinations with the three different concentrations of 24-Epi induced shootlets regeneration from either leaf or and stem explants.

Moreover, the frequency of direct regenerated shootlets produced from either leaf or stem explants was increased with graded increasing of 24-Epi concentrations. Where it recorded the maximum values (83 and 90%) with MS medium supplemented with 2 mg L^{-1} TDZ + 0.05 or 0.1 mg L^{-1} 24-Epi for leaf and stem explant, respectively. In addition, the maximum number of shootlets (12) was produced from stem explants cultured on MS medium supplemented with 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi.

However, there were insignificant differences among S_4, S_5 and S_6, which represented the lowest rates of regeneration at the same time. Also, the maximum number of leaves/shoot (23.5, 21) were recorded with MS medium supplemented with 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi for stem and leaf explants, respectively. While, there were insignificant differences between S_5, S_6 and S_4, S_5 for leaf and stem explants, respectively. Whereas, S_4 produced the minimum number of leaves/shoot (12.5). Moreover, the longest shootlet (9 cm) was produced on MS medium supplemented with 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi from stem explants. Whereas, 2 mg L^{-1} TDZ represents the shortest shootlet (6.20 cm) for stem explant.

In general, collected data pointed out that addition of 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi to MS medium greatly improved most vegetative growth criteria concerned to shootlets regeneration of *P. vulgaris* L. *via* direct organogenesis during the whole growth period in particularly for stem explants.

Effect of media composition and type of explants on accumulation of photosynthetic pigments in regenerated shootlets

The photosynthetic system is often highly sensitive to exogenous plant growth regulators. Therefore, the impact of MS medium fortified with different concentrations 0.025, 0.05 and 0.1 (mg L^{-1}) of 24-Epi alone or in combinations with 2 mg L^{-1} TDZ (regeneration media) on photosynthetic pigments content was examined. Where it had been evaluated the response of shootlets produced pigments from leaf and stem explants after exogenous of 24-Epi and TDZ applications. Chlorophyll *a*, *b* and carotenoids (mg/g FW) show significant differences between treatments, as shown in Table 2. MS medium supplemented with 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi induced highly significant increase in chlorophyll *a* content which recorded maximum value (311 mg/g FW) for shootlet produced from leaf explants. Moreover, the obtained results clearly indicated that, chlorophyll *a* content in shootlets produced from leaf and stem explants was increased with graded increasing of 24-Epi concentrations. Whereas, minimum value of chlorophyll *a* content (91 mg/g FW) was observed with MS medium supplemented with 2 mg L^{-1} TDZ for shootlets produced from leaf explant. However, shootlets produced from stem explants recorded the maximum increase in chlorophyll *b* content (575 mg/g FW) with MS supplemented with 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi. As well as, chlorophyll *b* content in shootlets derived leaf and stem explants was increased with increasing 24-Epi concentrations. Whereas, minimum value of chlorophyll *b* content (84 mg/g FW) observed with MS medium supplemented with 2 mg L^{-1} TDZ for shootlets produced from leaf explants. In addition, 24-Epi at the 0.025, 0.05 and 0.1 mg L^{-1} concentration significantly stimulated carotenoids. Where MS supplemented with 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi induced highly significant increase in carotenoids content which recorded the maximum value (996 mg/g FW). Furthermore, carotenoids content in shootlets produced from both leaf and stem explant was increased with graded increasing of 24-Epi concentrations. Whereas, minimum value of carotenoids content (372 mg/g FW) recorded with MS medium supplemented with 2 mg L^{-1} TDZ for shootlets produced from stem explants.

Effect of culture media composition on rooting of shootlets

The effect of MS medium supplemented with different concentrations of 24-Epi (0.025, 0.05 and 0.1 mg L^{-1}) alone or in combination with 0.5 mg L^{-1} IAA on rooting (%), number of roots, root length (cm) and roots dry weight (g/jar) of shootlets derived leaf and stem explants was investigated.

Table 3 clearly shows that MS medium supplemented with 0.5 mg L^{-1} IAA + 0.05 mg L^{-1} 24-Epi gave the maximum value for rooting percentage (71%) in case of shootlets produced from leaf explant. However, there were insignificant differences among R_3, R_6 and R_7. While, there were no responses to rooting on MS medium

Table 2. Effect of interaction between media composition and explants types on chlorophyll (a, b) and carotenoids contents of *P. vulgaris* L. cv. Brunca.

Culture media composition	Chlorophyll (a) mg/g FW	Chlorophyll (b) mg/g FW	Carotenoids mg/g FW
Shootlets of leaf explant			
S_0 = MS free growth regulators	-	-	-
S_1= MS + 0.025 mg L^{-1} 24-Epi	-	-	-
S_2= MS + 0.05 mg L^{-1} 24-Epi	-	-	-
S_3= MS + 0.1 mg L^{-1} 24-Epi	-	-	-
S_4= MS + 2 mg L^{-1} TDZ	91^{h}	84^{h}	421^{g}
S_5= MS + 2 mg L^{-1} TDZ + 0.025 mg L^{-1} 24-Epi	248^{e}	213^{e}	763^{d}
S_6= MS + 2 mg L^{-1} TDZ + 0.05 mg L^{-1} 24-Epi	300^{c}	298^{c}	815^{b}
S_7= MS + 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi	311^{a}	405^{b}	996^{a}
Shootlets of stem explant			
S_0 = MS free growth regulators	-	-	-
S_1= MS + 0.025 mg L^{-1} 24-Epi	-	-	-
S_2= MS + 0.05 mg L^{-1} 24-Epi	-	-	-
S_3= MS + 0.1 mg L^{-1} 24-Epi	-	-	-
S_4= MS + 2 mg L^{-1} TDZ	138^{g}	144^{g}	372^{h}
S_5= MS + 2 mg L^{-1} TDZ + 0.025 mg L^{-1} 24-Epi	198^{f}	185^{f}	461^{f}
S_6= MS + 2 mg L^{-1} TDZ + 0.05 mg L^{-1} 24-Epi	260^{d}	292^{d}	652^{e}
S_7= MS + 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi	310^{b}	575^{a}	678^{c}

(-) no shoots. Means having the same letters in a column were not significantly different at 0.05 level.

supplemented with 0.025 or 0.05 mg L^{-1} 24-Epi. In addition, there were insignificant differences among R_0, R_4 and R_5 that recorded the minimum value for rooting percentage (33%). On the other hand, concerning to shootlets resulted from stem explants, the best medium that produced the maximum rooting percentage (88%) was MS supplemented with 0.5 mg L^{-1} IAA + 0.1 mg L^{-1}24-Epi. As well as shootlets failed to roots formation on MS medium supplemented with 0.025 or 0.05 mg L^{-1} 24-Epi. Moreover, there were insignificant differences among R_3, R_4, R_5, R_6 and R_7. Furthermore, the minimum value of rooting percentage (36%) was obtained with R_0. The results demonstrated that MS medium supplemented with 0.5 mg L^{-1} IAA + 0.1 mg L^{-1}24-Epi was the most suitable medium for rootlets formation on shootlets of *P. vulgaris* L. cv. Brunca compared to all tested composition media (Figure 2A and B). Moreover, shootlets produced from stem explant recorded high response to rooting media compared to shootlets produced from leaf explant, where it recorded maximum rooting percentage (88%).

About number of roots and concerning to shootlets produced from leaf explant, there were insignificant differences among R_3, R_4, R_5, R_6 and R_7. Whereas R_3, R_6 and R_7 recorded the maximum number of roots formation (7.3). While R_0 gave the lowest number of roots formation (4.1) followed by R_4 (6.2). On the other hand, concerning to shootlets produced from stem explant, the best medium that produced the maximum number of roots (8.5) was R_7. Furthermore, there were insignificant differences among R_3, R_4, R_5 and R_6. Whereas, the minimum number of roots (5) was obtained with R_0.

Concerning the shootlets produced from leaf explant, there were insignificant differences among R_3, R_4, R_5, R_6 and R_7 that recorded the maximum root length (6.4 – 7 cm). While, the shortest root (3.2 cm) obtained with R_0. On the other hand, concerning to shootlets produced from stem explant, the longest root (9.4 cm) recorded with R_7 followed by R_3, which recorded (7.4 cm). Furthermore, there were insignificant differences among R_4, R_5 and R_6. Whereas, the shortest root (4.3 cm) was obtained with R_0 (MS free growth regulators).

In general, as mentioned before it can be concluded that shootlets derived stem explants showed a highly response to rooting media compared to shootlets derived leaf explants. Culturing of shootlets derived stem explants on R_7 medium recorded the highest values (88, 8.5 and 9.4) of rooting percentage, number of roots and roots length, respectively.

DISCUSSION

Legumes are not easily amenable to stable genetic transformation and hence, protocols for high throughput generation of transgenic legume plants are not available. In general, the difficulty for achieving efficient genetic transformation of legumes is related to their low responsiveness for *in vitro* regeneration (Arellano et al.,

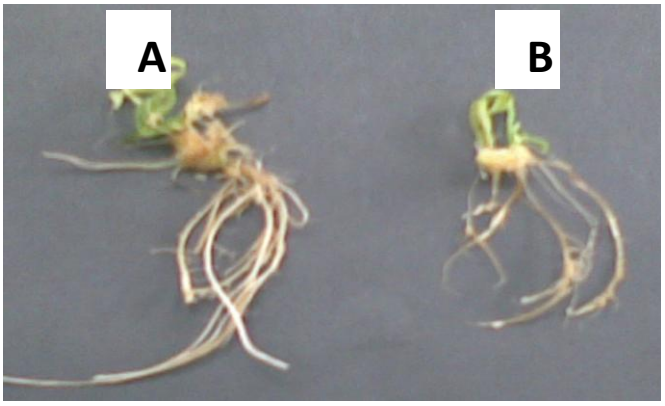

Figure 2. Rooting of regenerated shootlets derived from stem (A) and leaf (B) explants on MS medium supplemented with 0.5 mg L^{-1} IAA + 0.1 mg L^{-1} 24-Epi (5×).

Table 3. Effect of interaction between media composition and explants types on rooting, number of roots, root length and root dry weight of *P. vulgaris* L. cv. Brunca.

Culture media composition	Rooting (%)	Number of roots	Root length (cm)	Root dry weight (g/jar)
Shootlets derived leaf explant				
R_0= MS free growth regulators	33.3^{b}	4.1^{d}	3.2^{e}	0.03^{a}
R_1 = MS + 0.025 mg L^{-1} 24-Epi	-	-	-	-
R_2= MS + 0.05 mg L^{-1} 24-Epi	-	-	-	-
R_3= MS + 0.1 mg L^{-1} 24-Epi	70^{ab}	7.3^{b}	6.4^{c}	0.09^{a}
R_4 = MS + 0.5 mg L^{-1} IAA	33.3^{b}	6.2^{b}	6.4^{c}	0.07^{a}
R_5 = MS + 0.5 mg L^{-1} IAA+ 0.025 mg L^{-1}24-Epi	33.3^{b}	7.2^{b}	6.7^{c}	0.07^{a}
R_6 = MS + 0.5 mg L^{-1} IAA+ 0.05 mg L^{-1}24-Epi	71^{a}	7.3^{b}	6.4^{c}	0.09^{a}
R_7 = MS + 0.5 mg L^{-1} IAA+ 0.1 mg L^{-1}24-Epi	67^{a}	7.3^{b}	7^{c}	0.07^{a}
Shootlets derived stem explant				
R_0= MS free growth regulators	36.3^{b}	5^{c}	4.3^{d}	0.03^{a}
R_1 = MS + 0.025 mg L^{-1} 24-Epi	-	-	-	-
R_2= MS + 0.05 mg L^{-1} 24-Epi	-	-	-	-
R_3= MS + 0.1 mg L^{-1} 24-Epi	87^{a}	7.5^{b}	7.4^{b}	0.09^{a}
R_4 = MS + 0.5 mg L^{-1} IAA	68^{ab}	6.2^{b}	4^{c}	0.02^{a}
R_5 = MS + 0.5 mg L^{-1} IAA+ 0.025 mg L^{-1}24-Epi	70^{ab}	8^{b}	7^{c}	0.06^{a}
R_6 = MS + 0.5 mg L^{-1} IAA+ 0.05 mg L^{-1}24-Epi	70^{ab}	7.5^{b}	7^{c}	0.09^{a}
R_7 = MS + 0.5 mg L^{-1} IAA+ 0.1 mg L^{-1}24-Epi	88^{a}	8.5^{a}	9.4^{a}	0.07^{a}

Means having the same letters in a column were not significantly different at 0.05 level.

2009). Thus, in recent years, many research groups have been involved in establishing reliable regeneration procedures for economically important legumes, because it would be a primary step to facilitate gene introduction and improvement of the crop.

In vitro regeneration of *Phaseolus* spp. had been reported by organogenesis (Varisai Mohamed et al. 2006) or through somatic embryogenesis (Schryer et al., 2005). Although several protocols have been described in the literature for bean regeneration, development of an optimal *in vitro* culture system still remains a major challenge since this *P. vulgaris* and other species from the *Phaseolus* genus are recalcitrant for *in vitro* regeneration (Quintero-Jiménez et al., 2010). However, earlier studies on shootlet regeneration via direct organogenesis of *P. vulgaris* L. have been reported by several workers (Ahmed et al., 2002).

This study focused on the effects of 24- Epi alone or in combinations with TDZ on *in vitro* shootlets regeneration in *P. vulgaris* L. cv. Brunca. The naturally occuring

Brassinosteroids (BRs) are a new group of plant hormones with significant growth-promoting activity. Application of BRs significantly stimulated adventitious bud formation from hypocotyl segments of cauliflower cultured *in vitro* in the light**.** Additive effect of BRs with cytokinins was also demonstrated on shoot regeneration from cauliflower hypocotyl segments (Sasaki, 2002). BRs affect regeneration potentials positively and their applications could be useful for regeneration of recalcitrant genotypes (Lu et al., 2003).

EBR (24-epibrassinolide) was suggested to be able to induce the activity of cytokinin, BRs have also been reported to be involved on branching responses and changing endogenous cytokinin levels in various plant species (Pereira-Netto et al., 2003). The efficacy TDZ on direct multiple shoot formation from *P. vulgaris* L, which is attributed that TDZ mimics the effects of cytokinins on organogenesis. Furthermore, TDZ may be involved in the synthesis and/or accumulation of cytokinins in plant tissue culture (Carvalho et al., 2000). In this regard, TDZ is considered one of the most promising cytokinin for shoot induction in legumes (Jayanand et al., 2003; Tewari et al., 2004; Kiran et al., 2005; Anwar et al., 2008, 2010). TDZ was most effective inducing healthier shoots when used at lower concentration (~2.2 mg L^{-1}) compared to higher concentrations (Jiang et al., 2005; Anwar et al., 2008, 2010).

In the present study, the obtained results clearly demonstrated that 24-Epi (at the different concentrations used) induced shootlets regeneration via direct organogenesis of *P. vulgaris* L. cv. Brunca when combined with 2 mg L^{-1} TDZ. The highest response was recorded with 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi. These findings are similar to those of Anwar et al. (2008 and 2010) and Jiang et al. (2005). Concerning regeneration from root explants, it is observed that root explants completely failed to shootlets regeneration. Therefore, Yoshida (2002) observed that too much contact of the explants into the medium may inhibit adventitious shoot formation in soybean. This may suggest that there is a considerable variation existed between the different types of explants in their ability to form shoots.

Furthermore, 24-Epi enhanced the accumulation of photosynthetic pigments content (chlorophyll *a*, *b* and carotenoids) in the regenerated shootlets. These results are in agreement with the results of Janeczko et al. (2007) and Wang and Zeng (1993). In addition, 24-Epi promoted rooting of *in vitro* regenerated shootlets. These results coincided with the results of Vardhini and Rao (2003), Kartal et al. (2009) and Vardhini et al. (2012).

Conclusion

It could be concluded that a simple, reliable and efficient regeneration system of *P. vulgaris* from stem explants was established. Thus, shoot regeneration via direct organogenesis is likely to be good for genetic transformation of this crop. The procedure presented in this study yields efficient adventitious shoots regeneration from stem-segment explants. The high frequency of shootlets (90%) may be very advantageous for a variety of purposes, including both classical and molecular breeding of *P. vulgaris*.

Conflict of Interests

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

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Full Length Research Paper

Production of extracellular lipase by a new strain *Staphylococcus aureus* NK-LB37 isolated from oil contaminated soil

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A total of 20 bacterial isolates were obtained by screening using tributyrin agar medium. Among them the isolate which exhibited greater clearance zone and higher lipase activity was subsequently screened using spirit blue agar and rhodamine B agar medium. Based on morphological, biochemical and 16S rRNA sequence analysis, the potent isolate was identified as *Staphylococcus aureus*. The lipase production of the isolate was increased by improving the conditions of production medium. Maximum lipase production (8.11 U/ml) was achieved when 2% punnakka oil was utilized as sole carbon source at pH 7.0 and 37°C after 2 days of incubation. Addition of 3% tryptone as nitrogen source and 0.01% $MgCl_2$ resulted in a significant increase of lipase production (10.73 U/ml). The lipase production was slightly enhanced in the presence of 20% n-propanol and highly stable in the presence of n-butanol, toluene and n-hexane. The study resulted in isolation and production of inducible, mesophilic and solvent tolerant lipase with industrial potential.

Key words: *Staphylococcus sp*., tributyrin agar, 16S rRNA , medium optimization, solvent-tolerant lipase.

INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyze the hydrolysis of ester linkages of triglycerides at water-oil interface (Gupta et al., 2011). Apart from hydrolysis, some lipases can catalyze reverse reactions including synthesis of esters by esterification, transesterification and interesterification (Franken et al., 2011). Existence of lipase producing microorganisms have been found in diverse habitats such as industrial wastes, vegetable oil processing units, dairies, soil contaminated with oil, compost heap, hot springs etc (Sztajer et al., 1988; Wang et al., 1995).

Although lipases are widely found in animals, plants and microbes, microbial lipases have gained special industrial attention due to their selectivity, stability and substrate specificity (Treichel et al., 2010). Bacterial lipases are mostly extracellular and are greatly influenced by nutritional and physico-chemical parameters such as temperature, pH, nitrogen and carbon sources (Gupta et

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Abbreviations: TBA, Tributyrin agar; **SBA,** spirit blue agar; **RBA,** rhodamine B agar; **p-NPP,** p-nitrophenylpalmitate.

al., 2004).Generally, organic solvents are known to have detrimental effect on microorganisms and so they lose their activity and cease growing (Ogino et al., 1994). However, certain microbial lipases have gained importance due to their ability of being active in the presence of organic solvents (Sellek et al., 1999). Only a few species of bacteria have been exploited for the production of solvent tolerant lipases.

Recently due to huge variation in industrial applications, the availability of lipases with specific characteristics is still required to be explored. Further screening may lead to isolation of novel lipases with desired properties. Therefore, the present study has been attempted to screen and identify a new bacterial strain for the production of extracellular solvent tolerant lipase. It was further investigated in liquid medium in order to optimize the lipase production conditions.

MATERIALS AND METHODS

p-Nitrophenylpalmitate was obtained from Sigma Aldrich (USA). Microbiological media such as potato dextrose agar, tributyrin agar, spirit blue agar, rhodamine B and sodium deoxycholate were purchased from HiMedia Laboratories, Mumbai, India. All other chemicals utilized in the study were of analytical grade.

Sample collection

Soil samples were collected from oil contaminated areas of different oil mills in and around Coimbatore, Tamilnadu in sterile containers. The samples were transferred to the laboratory and stored at 4°C until its needed.

Screening and selection of lipase producing bacteria

Ten gram of soil sample was suspended in 100 ml of sterile distilled water by shaking (160 rpm) at 37°C for 4 h. Then the soil samples were filtered using sterile cheese cloth and the filtrate was used for isolation of bacteria. The isolation process was performed by serial dilution (10^{-1} to 10^{-6}) of soil sample (200 µL) on tributyrin agar selective medium (Booth,1971). The plates were incubated at 37°C for 2 days for the development of bacterial colonies. Twenty colonies showing clear zones around them were individually spot inoculated on tributyrin agar medium. Based on zone of clearance and lipase activity, the isolate LB5 was selected for further studies.

The efficiency of the isolate LB5 to produce lipase was confirmed by further screening using spirit blue agar and Rhodamine B agar media. The colonies showing blue color zones on spirit blue agar was subsequently streaked on Rhodamine B agar medium according to the method described by Kouker and Jaeger (1987). Lipase production was detected by irradiating the plates with UV light at 350 nm. Bacterial colonies having lipolytic activity showed orange fluorescent halo around the colony. All screening media used in the study were adjusted to pH 7.0 and plates were incubated at 37°C for 2 days. The pure culture was subcultured on nutrient agar slants and stored at 4°C for further use.

Identification of bacterial isolate

The morphological and biochemical characterization of the isolate LB5 was performed according to the Bergey's Manual of Determinative Bacteriology (Holt et al., 1989). The identification was further confirmed by the 16S rRNA gene sequencing analysis. Briefly, genomic DNA was extracted from the culture LB5 using GeNei Ultrapure bacterial genomic DNA isolation kit (KT162). The DNA was amplified by PCR using 16S rRNA gene specific primers (Forward primer: 5' AGA GTT TGA TCM TGG CTC AG 3' and Reverse primer: 5' TAC GGY TAC CTT GTT ACG ACT T 3'). The PCR program comprised initial denaturation at 94°C for 5 mins, 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1.30 min and a final extension step at 72°C for 10 min using Thermal cycler ABI 2720. The amplified PCR product was confirmed by agarose gel electrophoresis (1.0%). The amplicon (1500 bp) was purified by PCR purification kit (KP153). The PCR product was sequenced by ABI 3100 XL Genetic Analyzer using Big Dye Terminator version 3.1"Cycle sequencing kit. The 16S rRNA gene of the sample was compared with NCBI-GenBank and RDP databases. Sequences were aligned using the Clustal W program. A distance matrix was generated based on sequence homology using Kimura-2 Parameter and phylogenetic tree was constructed using Neighbour joining method.

Lipase production

The bacterial culture was grown in 250 ml Erlenmeyer flask containing 50 ml of basal medium of following composition $CaCl_2.2H_2O$, 0.01%; $MgSO_4.7H_2O$, 0.04%; $FeCl_3.6H_2O$, 0.04%; pH 7.0 (Eltaweel et al., 2005). Olive oil (1% v/v) and peptone (1% w/v) were used as sole source of carbon and nitrogen respectively. The flasks with medium were inoculated with 1×10^6 spore suspension and was incubated at 37°C for 2 days under shaking (200 rpm). After incubation, the culture was centrifuged at 10000 rpm for 20 min at 4°C .The supernatant obtained was used as crude enzyme source for lipase assay

Lipase assay

Lipase activity was assayed using pNPP as substrate with some modifications (Winkler and Stuckmann, 1979). In brief, 10 ml of isopropanol containing 30 mg of pNPP was mixed with 90 ml of 0.05 M phosphate buffer (pH 7.6) containing 200 mg of sodium deoxycholate and 100 mg of gum arabic. A total of 2.4 ml reaction mixture was added with 0.1 ml of crude enzyme source and incubated at 37°C for 15 min. The release of p-nitrophenol was determined by measuring the absorbance at 410 nm against an enzyme free control as blank. One unit of lipase activity (U) was defined as the amount of enzyme releasing 1 µmol of p-nitrophenol per minute under the assay conditions. All the experiments were performed in triplicates and the mean values were taken for analysis.

Optimization of cultural conditions for lipase production

The incubation time required for the maximum lipase production was studied by measuring the lipase activity at different time intervals of 12 to 60 h. In order to determine the optimum pH of the culture, pH of the medium was adjusted to 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0. The optimal temperature for lipase production was studied at different temperatures (25, 30, 37, 40, 45 and 50 °C). Variation in lipase production in response to carbon sources (jatropha oil, neem oil, punnakka oil, cotton seed oil, karanja oil, castor oil) was studied by replacing the olive oil in the basal medium at a concentration of 1% (v/v). The organic nitrogen sources were incorporated to the basal medium at 1% (w/v) by replacing peptone which include yeast extract , tryptone, corn steep liquor, casein and soyabean meal. Inorganic nitrogen sources (0.2% w/v) added to the medium includes ammonium tartrate, ammonium carbonate, ammonium nitrate and ammonium dihydrogen phosphate.

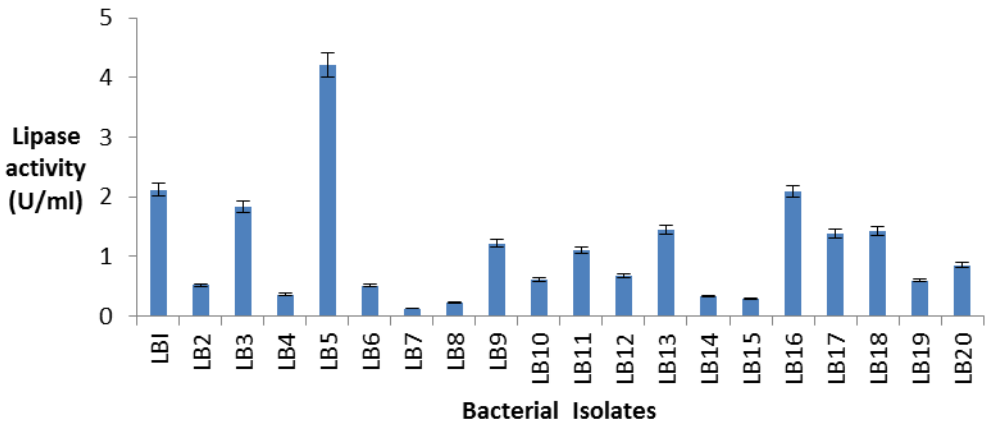

Figure 1. Lipase activity of different bacterial isolates.

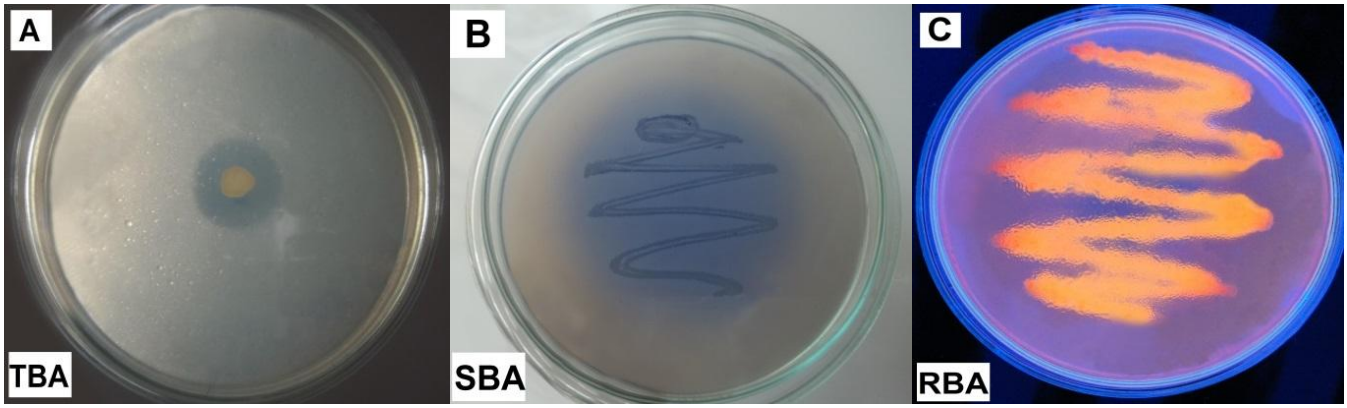

Figure 2. Growth of the potent bacterial isolate LB5 on different lipase screening medium. **A.** Tributyrin agar (TBA). **B.** Spirit blue agar (SBA); **C.** Rhodamine B agar (RBA).

The effect of different chloride salts of metal ions (Cu^{2+}, Ni^{2+}, Mg^{2+}, K^{+}, Co^{2+}, Zn^{2+} and Mn^{2+}) at 0.01% concentration was evaluated to observe the changes in lipase production.

Effect of organic solvents on crude enzyme stability

The influence of various organic solvents (n-propanol, n-butanol, methanol, ethanol, toluene and n-hexane) at 20% concentration was studied. One ml of organic solvent was added to 4 ml of crude enzyme and incubated at 37°C for 1 h under shaking condition (160 rpm) to facilitate proper mixing of enzyme and solvent. The enzyme stability was designated as residual activity under the standard assay conditions.

RESULTS AND DISCUSSION

Screening and selection

Several methods have been proposed for screening of lipase production but there is still more scope for finding lipases with novel and specific properties through screening using tributyrin agar and plate assay (Rohit et al., 2001). Therefore the present study employs three screening strategies to study the efficiency of the new bacterial isolate for lipase production.

Twenty bacterial isolates (LB1-LB20) were found to produce lipase as indicated by the clearance zone around the colony on the tributyrin agar plates. Among them, LB5 showed the maximum zone of clearance (11 mm) and higher lipase activity (4.21 U/ml) as shown in the Figure 1. The isolate LB5 was further screened using spirit blue agar and rhodamine B agar to determine its lipase producing efficiency which too was found to be positive (Figure 2)

Identification of potent bacterial isolate

The isolate LB5 was identified as *Staphylococcus sp*. based on its morphological and biochemical characterization (Table 1 and Figure 3). The identification was further confirmed by molecular characterization.

Molecular characterization

The molecular identification was carried out by the isola-

Table 1. Morphological and Biochemical characterization.

Test Name	LB5
Colony morphology	
Size	Small
Form	Circular
Shape(Negative staining)	Cocci
Arrangement	Clusters
Margin	Entire
Elevation	Raised
Density	Opaque
Texture	Smooth
Pigmentation	Creamy white
Gram reaction	Positive
Biochemical Tests	
Indole	-
Methyl red	-
Vogues-proskauer	-
Citrate Utilization Test	-
Catalase	+
Oxidase	-
Urease	-
Nitrate Reduction Test	+
Glucose Fermentation	+
Sucrose Fermentation	+
Lactose Fermentation	+
Starch Hydrolysis	+
Casein Hydrolysis	+
Gelatin Hydrolysis	-
Cellulose Hydrolysis	+
Tween-80 Hydrolysis	+
Motility Test	Non-motile
Coagulase Test	+

tion and sequencing of the 16S rRNA of the isolate LB5 (Figure 4). The 16S rRNA gene sequence of isolate LB5 contains 1505 bp. The sequence was compared with 10 different closely related sequences available in GenBank and RDP databases. To analyze the phylogenetic relationship of strain LB5, 16S rRNA gene sequence was determined and a dendrogram was constructed. The phylogenetic analysis revealed that the strain LB5 had highest homology with *S. aureus strain SR-05-03* (GenBank Accession Number: JQ247719.1) followed by *S. aureus strain: NBRC 12* (GeneBank Accession Number: AB680330.1). Based on these results, the isolate LB5 was designated as *S. aureus* (Figure 5).The 16S rRNA gene sequence of isolate LB5 has been deposited in the NCBI - GenBank database under the Accession Number KF152912

Optimization of parameters for lipase production

Bacterial lipases are influenced by the composition of the growth medium, cultivation conditions and many physico-chemical (pH and temperature) and nutritional factors (carbon, nitrogen and lipid sources) (Jaeger et al., 1994). The influence of the components of the growth medium on microbial lipase production varies from one organism to another. The strategy adopted to optimize the parameters for improved lipase production was one-factor-at-a-time method

Effect of incubation time on lipase production

The lipase production was observed during a period of 12 to 60 h at 12 h interval. Substantial lipase production was detected after 24 h in late log phase. Maximum lipase production was obtained at 48 h at the early stationary phase (Figure 6A). Further incubation beyond 48 h was found to have negative effect on lipase production. This may be due to the release of by-products during decline phase. This finding is in accordance with Pogaku et al. (2010) who observed that 48 h of incubation time to be desirable for *Staphylococcus* sp. Lp12

Effect of pH

The pH of the growth medium is one of the important parameters affecting microbial cell growth and biochemical metabolism. The lipase yield was observed in pH range 7.0 to 8.5, the optimum being pH 7.0 (Figure 6B). Lipase production dropped significantly beyond pH 8.5. The finding agreed with Sirisha et al. (2010) who observed maximum lipase activity at pH 7.0 by *Staphylococcus sp*.

Effect of temperature

Lipase production was maximum at the optimum temperature of 37°C. Lipase production showed gradual increase with increase in temperature from 25 to 37°C. And with further increase of temperature beyond 40°C, the enzyme production decreased (Figure 6C). Various industrial application of microbial lipases for the hydrolysis of glycerides, inter-transesterification of fatty acid moieties etc., basically involved the use of mesophilic enzymes.

Effect of carbon source

Lipase production by the isolate LB5 cleaved all the tested non-edible oils with the highest affinity to punnakka oil (7.36 U/ml) followed by karanja oil (Figure 7A). While all the other oils also showed considerable amount of enzyme production indicating the inducible nature of the enzyme most bacterial lipases are generally induced in medium that contains the proper fatty acids and oils (Immanuel et al., 2008; Sharma et al., 2009).

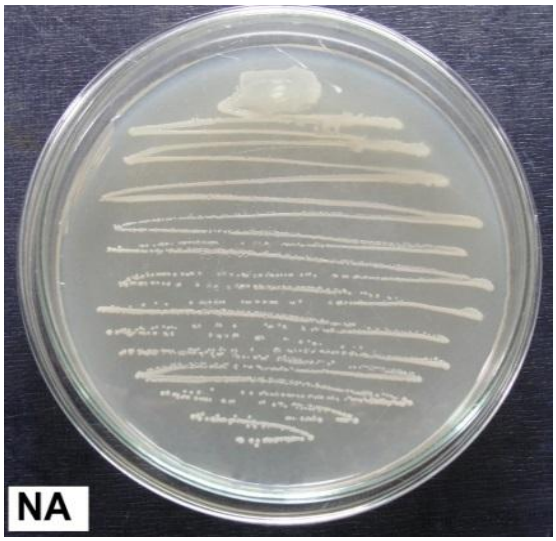

Figure 3. Growth of the bacterial isolate LB5 on nutrient agar (NA).

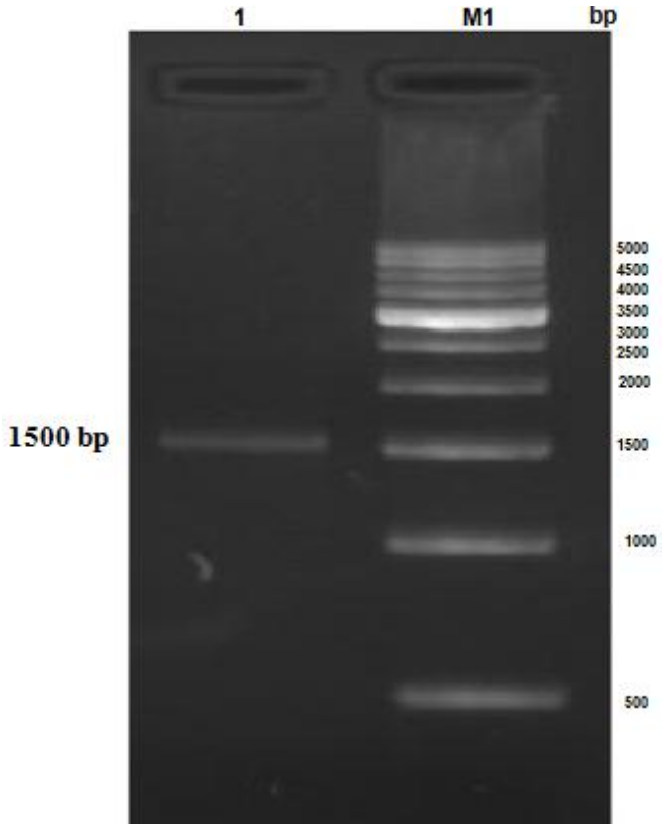

Figure 4. Agarose gel electrophoresis of the amplified PCR product. Lane 1 , sample (Isolate LB5); lane M1, Stepup^{TM} 500bp DNA ladder.

Many reports have shown that natural oil stimulate lipase production (Abdel-Fattah, 2002; Kaushik et al., 2006; He and Tan, 2006). In attempts to optimize the concentration of punnakka oil, it was observed that upto 2% (v/v) gave maximum lipase production (8.11 U/ml) (Figure 7B). Higher levels of punnakka oil showed a deleterious effect on lipase production which might be due to the inhibition by fatty acid concentration which was liberated during the hydrolysis of triglycerides.

Effect of nitrogen source

Besides carbon source, the type and concentration of nitrogen source in the medium also play an important role in the synthesis of enzymes. Among the different organic nitrogen sources used, tryptone was found to be the most suitable nitrogen source showing maximum lipase production (9.82 U/ml) (Figure 8A). Further studies conducted by incorporating tryptone at different concentrations showed that addition of 3% (w/v) tryptone stimulated lipase production (10.33 U/ ml) (Figure 8B). All the tested inorganic nitrogen sources were found to exhibit inhibitory effect on lipase yield indicating that organic nitrogen sources were preferred to inorganic nitrogen sources for lipase production (Figure 9A). So the inorganic nitrogen sources were not included in the optimized medium. Generally microorganisms exhibit high yield of lipase when organic nitrogen sources were used in the medium (Mobarak-Qamsari et al., 2011)

Effect of metal ions

Metal ions enhance the enzyme activity and confer thermostability to them (Chakraborty and Paulraj, 2008). Many enzymes in the presence of metal ions facilitate the maintenance of their active structures (Sharma et al., 2002). Different lipases show different response to metal ions. The presence of $MgCl_2$ in the culture medium was found to stimulate lipase production (10.76 U/ml) (Figure 9B). Other ions such as Mn^{2+} and K^{+} slightly inhibited lipase production. This finding is in accordance with Kalpana et al. (2013) who reported that *Staphylococcus* TUL1 showed maximum lipase production in the presence of Mg^{2+}. Heavy melals such as Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+} strongly inhibited lipase production. Generally lipase activity in the presence of heavy metals like Co^{2+}, Ni^{2+}, Hg^{2+} and Sn^{2+} was inhibited (Patkar and Bjorkling, 1994).

Effect of organic solvents on the lipase stability

Lipases are diverse in their sensitivity to organic solvents (Raku et al., 2003). In this study, the stability and activity of various organic solvents were tested at 20% (v/v) concentration as depicted in Figure 10. Organic solvent n-propanol slightly enhanced the lipase activity. The crude lipase was stable in n-butanol, n-hexane and toluene with residual activity of 79.3, 88.6 and 90.0% respectively, whereas methanol and ethanol showed negative effect on lipase activity. Therefore, it can be inferred that the

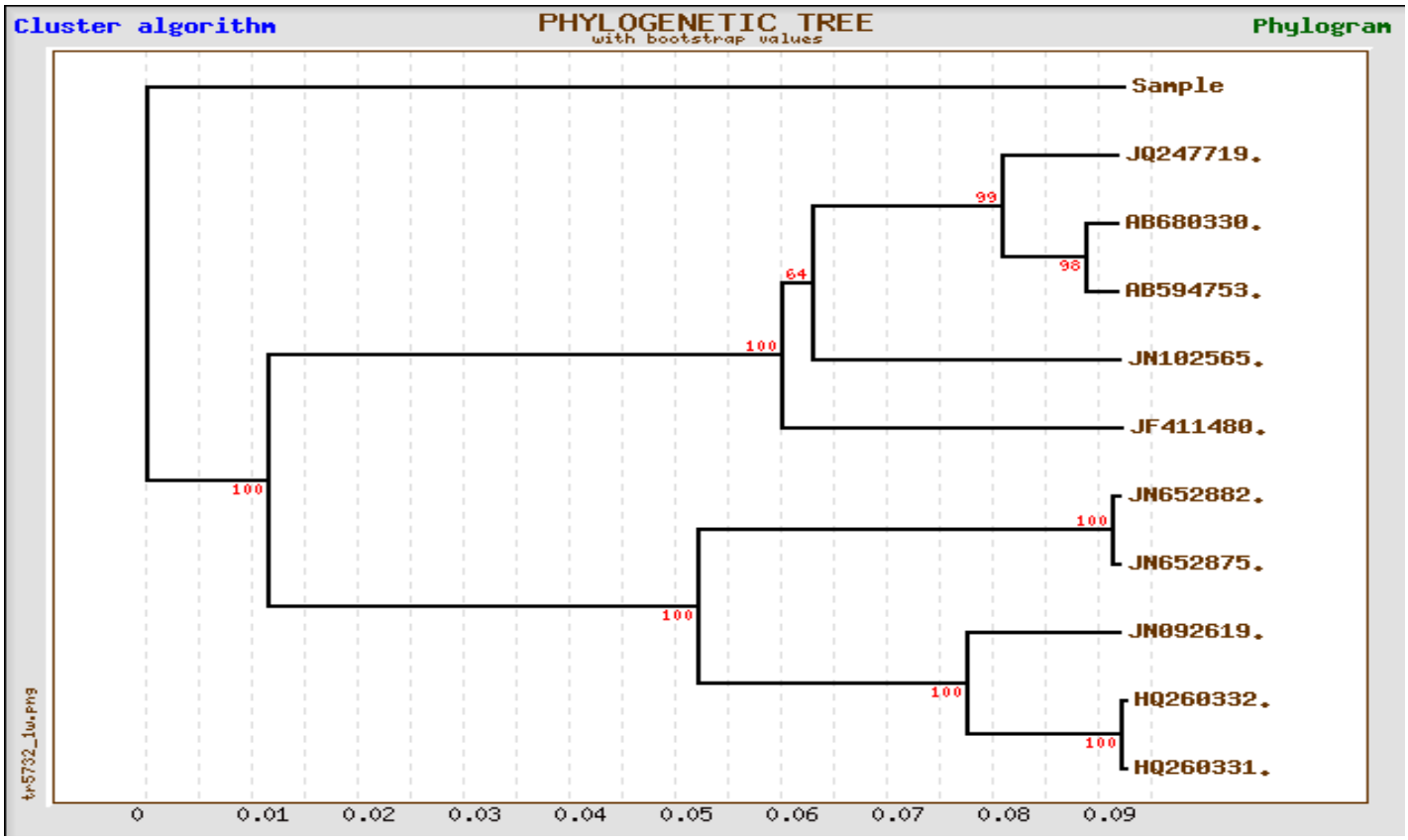

Figure 5. Phylogenetic tree based on 16S rRNA gene sequence showing the relationships between the Sample (isolate LB5) and related members of the genus *Staphylococcus.*

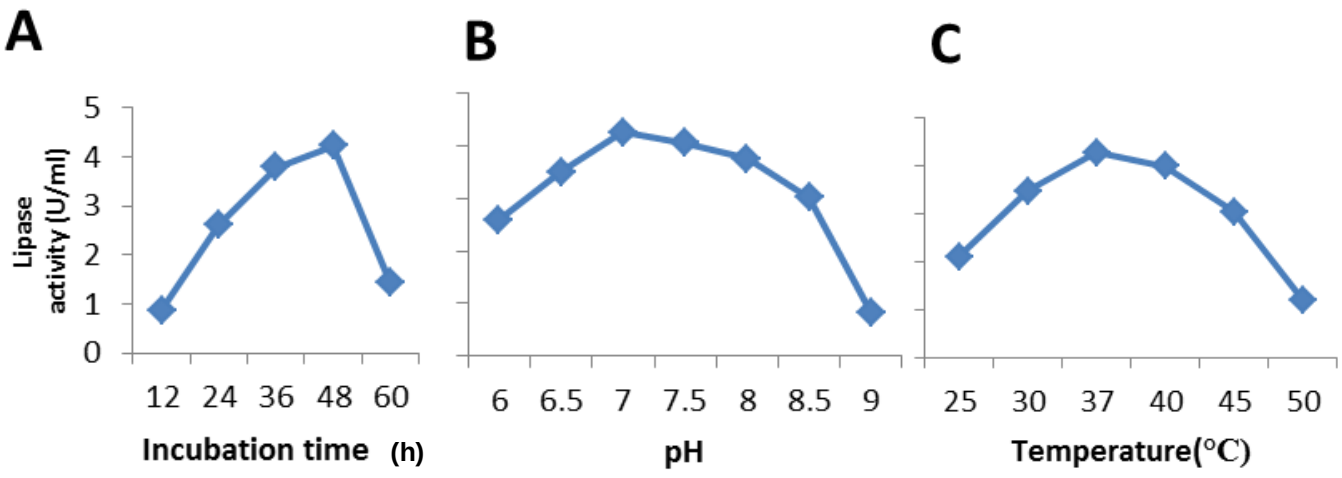

Figure 6. Effect of Incubation time (A) , pH (B) and temperature(C) on lipase production.

strain NK-LB37 exhibited different extents of tolerance to various organic solvents. Organic solvent tolerant lipases are effective catalysts in the transesterification reactions and synthesis of biopolymers (Dizge et al., 2009; Singh et al., 2010).

Conclusion

Lipases occupy a prominent place among biocatalysts with a wide spectrum of industrial applications. Considering this, in the present study, the production of lipase

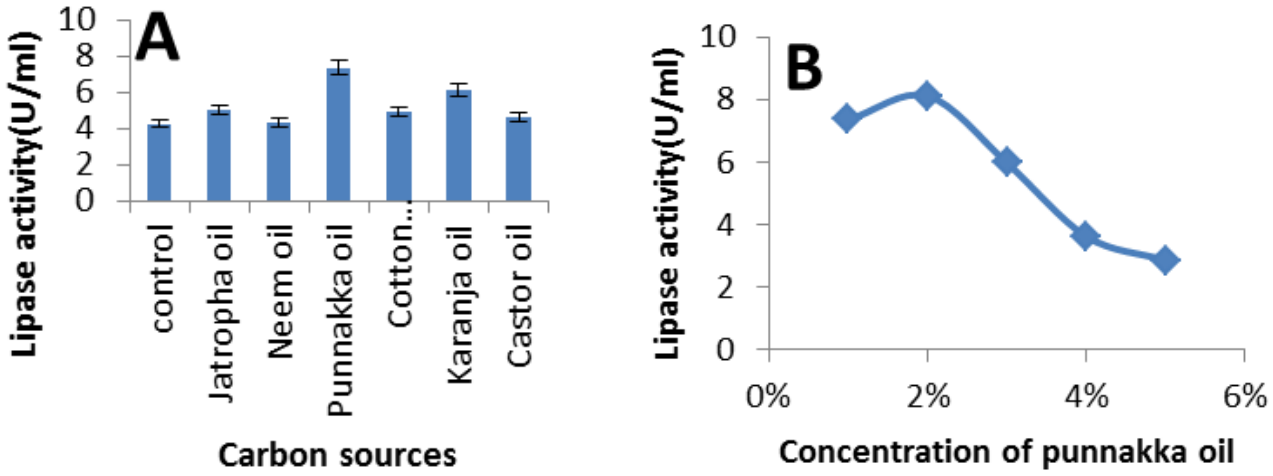

Figure 7. Effect of carbon sources (A) and concentration of punnakka oil (B) on lipase production.

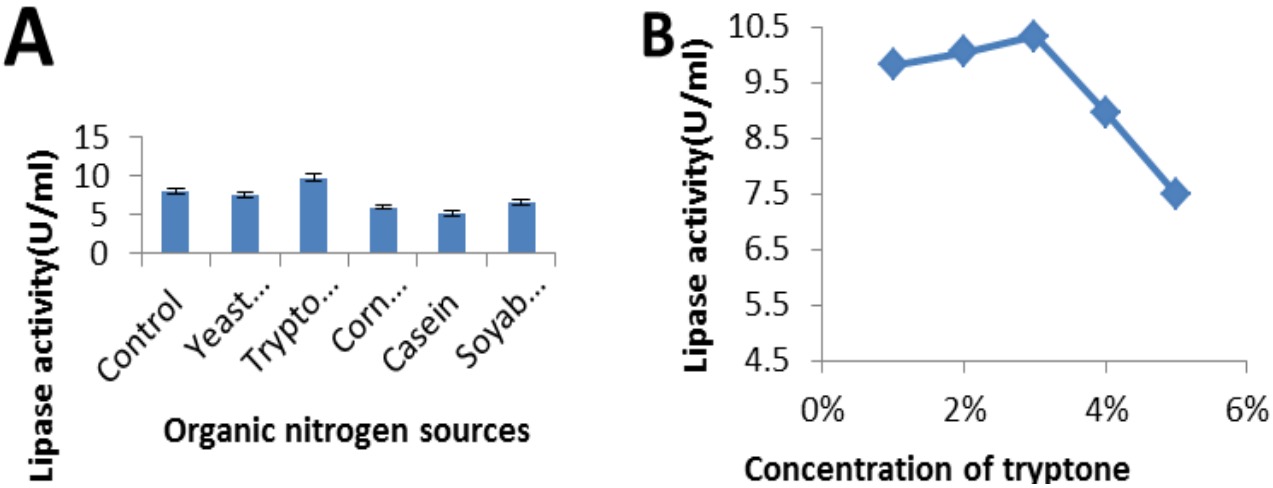

Figure 8. Effect of organic nitrogen sources (A) and concentration of tryptone (B) on lipase production.

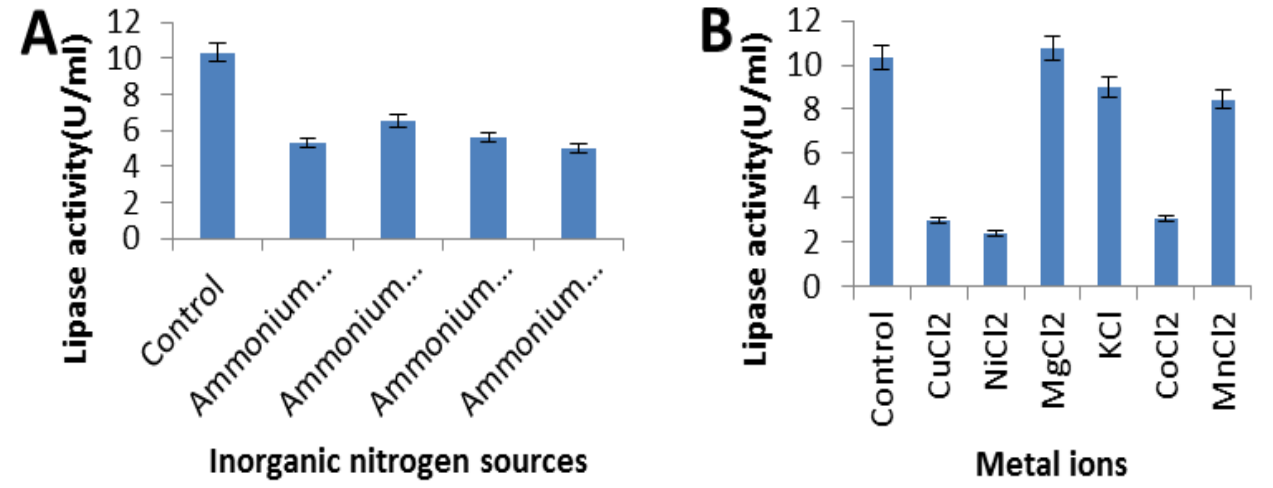

Figure 9. Effect of inorganic nitrogen sources (A) and metal ions (B) on lipase production.

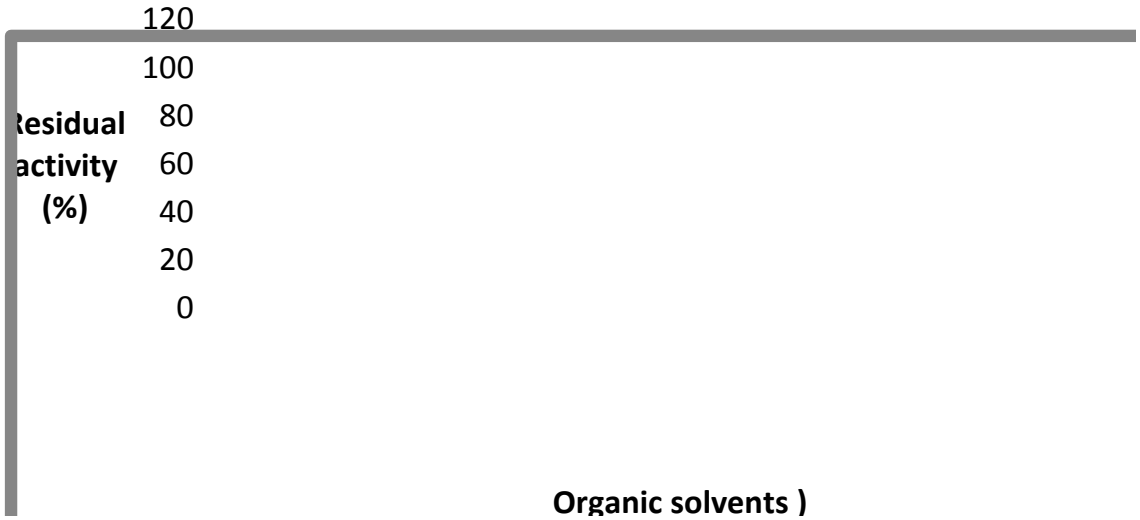

Figure 10. Effect of organic solvents on lipase stability. Residual activity was compared with the control without organic solvent. 100% was assigned to the control sample.

from a new isolate *Staphylococcus sp*. was optimized. The final optimized medium resulted in an overall 2.5 fold enhanced lipase production. This study presents significant observation on the use of 2% punnakka oil and 3% tryptone as carbon and nitrogen sources to significantly enhance the synthesis of lipase. It was also observed that the crude lipase was stable in various organic solvents with slight increase in activity when n-propanol was added to the basal medium. Therefore based on the above characteristics, this lipase may find applications in biodiesel production, oleochemical industry, polymer synthesis and triglycerides synthesis.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Full Length Research Paper

Amino acids analysis during lactic acid fermentation by single strain cultures of lactobacilli and mixed culture starter made from them

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The interactions among *Lactobacillus salivarius*, *Pediococcus acidilactici* and *Lactobacillus plantarum* in utilizing the amino acids of MRS media were investigated. *L. salivarius* alone showed relatively good assimilation of various amino acids that existed at only a little amounts in MRS media (Asn, Asp, Cit, Cys, Glu, His, Lys, Orn, Phe, Pro, Tyr, Arg, Ile, Leu, Met, Ser, Thr, Trp and Val), whereas Ala and Gly accumulated in *L. salivarius* cultures. *P. acidilactici*, in contrast, hydrolyzed the proteins found in the medium to liberate various amino acids; it utilized Cit, Cys, and Gly for growth, while promoting the accumulation of Ala, Asn, Glu, His, Lys, Orn, Phe, Pro, Tyr, Arg, Ile, Leu, Met, Ser, Thr, Trp, and Val. Similar to *L. salivarius, L. plantarum* showed relatively good assimilation of various amino acids that existed at only trace amounts in MRS medium (Ala, Asn, Asp, Cit, Cys, Glu, Gly, His, Lys, Phe, Pro, Tyr, Arg, Ile, Leu, Met, Ser, Thr, Trp, and Val). Single-strain cultures of *L. salivarius* and *P. acidilactici* exhibited high levels of growth and lactic acid synthesis, whereas single-strain cultures of *L. plantarum* manifested the lowest cell growth and lactic acid synthesis. The unique individual abilities of the three LAB strains to utilize amino acids for growth and lactic acid production were employed to create a mixed culture that showed enhanced cell growth and lactate production. The results from our mixed culture (*L. salivarius* and *P. acidilactici*) experiments suggested that the growth of *L. salivarius* was stimulated by the amino acids produced by *P. acidilactici*, enhancing lactic acid production. Mixed cultures were tested at different inoculation proportions (1:1, 2:1, 5:1 and 9:1; given as OD_{600} ratios of *L. salivarius*: *P. acidilactici*), and the highest cell growth and lactic acid production values were obtained for the 2:1 ratio.

Key words: Amino acids, single and mixed culture, *Lactobacillus salivarius*, *Pediococcus acidilactici*, *Lactobacillus plantarum*

INTRODUCTION

Lactic acid bacteria (LAB), which are used for the fermentation of various foods and beverages, are

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facultative anaerobic bacteria that convert the available sugar into lactic acid as the major end product. Probiotics are live microorganisms that are present in food and are capable of modifying the gut microflora to improve human (or animal) health. The probiotic bacteria are found among the genera *Lactobacillus*, *Bifidobacterium*, *Enterococcus* and *Bacillus*. Due to the U.S. Food and Drug Administration's restrictions on the use of antibiotics in animals raised for food, researchers have sought alternative strategies to control infectious diseases in animals such as poultry. One feed supplement that can enhance performance and protect animals from microbial infection is the large-scale application of probiotic bacteria in animal feed (Gaggia et al., 2010). Thus, there is a market for huge LAB biomasses (Schiraldi et al., 2003; Briens et al., 2008; Garvie, 1967), and new production strategies are needed.

LAB of the genus *Lactobacillus* are fastidious bacteria with numerous growth requirements, including the need for rich media containing compounds such as amino acids, peptides, vitamins and nucleic acids (Narayanan et al., 2004; Dumbrepatil et al., 2008; Mills and Thomas, 1981). LAB initiates growth faster and reach higher cell densities in complex media containing fundamental nitrogen components, such as amino acids or low-molecular-weight proteins. The majority of amino acids are either stimulatory or essential for their growth (Garvie, 1967). De Man, Rogosa and Sharpe (MRS) medium is commonly used to grow *Lactobacillus*.

The amino acid requirements and lactic acid production activity of LAB can be optimized by using mixed-strain cultures, wherein strains that produce certain amino acids can compensate for their use by other strains that have higher production activity. Upon noticing that co-cultures of *Lactobacillus acidophilus* plus *Kluyveromyces fragilis* yielded higher lactic acid production rates and values than those seen with either strain alone, Yu et al. (1987) investigated the cross-promoting action of two strains on lactic acid fermentation by examining different sugars (for example sucrose, raffinose and stachyose in soy milk). In this study, to examine the possible synergetic effects between strains of *Lactobacillus* in a co-cultured system, we first monitored the changes in free amino acid levels during single-strain cultures. We assessed the amino acid release, amino acid consumption, cell growth, and lactic acid production in single-strain cultures of LAB and mixed-strain cultures composed of different ratios of *Lactobacillus salivarius* and *Pediococcus acidilactici* starter cultures. This study provides important baseline information for the development of promising and effective means for commercial culturing of LAB.

MATERIALS AND METHODS

Bacterial strain and preparation of inocula

L. salivarius (Ls 21), *P. acidilactici* (Pa 175) and *L. plantarum* (Lp 177), which were previously isolated from a pig farm in Korea (Yun et al., 2008), were used in the present study. Stock cultures of *L. salivarius*, *P. acidilactici*, and *L. plantarum* were maintained at -80°C in MRS medium containing 40% (v/v) glycerol. Precultures were prepared by inoculating 1 ml of frozen stock into 50 ml of MRS medium, followed by incubation at 37°C for 16 h. Cultures (single- or mixed-strain) were prepared by inoculating 1 ml (10^6 CFU/ml) of preculture into 100 ml of MRS medium in a 500 ml Erlenmeyer flask, followed by incubation at 37°C for 16 h with shaking at 150 rpm. The bacteria were grown under anaerobic conditions in the glucose-containing MRS medium. The medium composition per liter was as follows: (a) 10 g peptone, 10 g beef extract, 5 g yeast extract, 3 g diammonium citrate, 5 g sodium acetate, 1 g Tween, 2 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2 g $MnSO_4 \cdot 4H_2O$; and (b) 15 g glucose. Components (a) and (b) were autoclaved separately and aseptically mixed together immediately prior to cultivation. For the mixed-strain experiments, cultures were inoculated with 1 ml of precultures mixed at OD ratios ranging from 1:1 to 9:1 (*L. salivarius*: *P. acidilactici*). Each experiment was repeated three times.

Analysis of amino acid concentration

Free amino acids were quantified from culture supernatants taken at 8 h. The culture samples were filtered with a membrane (0.45 μm, GS; Millipore, [Bedford, U.S.A]) and hydrolyzed with 6 M HCl for 24 h at 110°C under a vacuum, and amino acid contents were measured using a Hitachi model L8800A automated amino acid analyzer (Hitachi, Japan).

Other analytical methods

Bacterial growth was monitored by spectrophotometric measurement at 600 nm. To assess lactic acid production, the fermentation broth was centrifuged at 20,000 x *g* for 10 min, the supernatant was collected, and lactic acid concentrations were assessed using an HPLC apparatus equipped with a refractive index detector (Agilent, U.S.A). The utilized column was an Aminex HPX-87H (Bio-Rad Co., USA), and chromatography was performed at 40°C using 0.01 N H_2SO_4 as the eluent at a flow rate of 0.6 ml/min.

RESULTS AND DISCUSSION

Amino acid utilization/production of individual *Lactobacillus* strain

The free amino acid levels in non-fermented MRS media (0 h) and those subjected to fermentation by the three individual *Lactobacillus* strains at 37°C (8 h, the end of the exponential growth phase) are presented in Table 1. Fermentation of MRS medium by *L. salivarius* decreased the contents of all amino acids except alanine and glycine, all of which are essential growth factors for this bacterium. In contrast, the levels of alanine and glycine increased compared to their levels in unfermented MRS broth. These findings confirm that *L. salivarius* released alanine and glycine, which are non-essential for the growth of this bacterium.

P. acidilactici increased the amino acid content of the medium, releasing numerous (non-essential) amino acids to the medium, including all amino acids except citrulline, cysteine and glycine. Thus, the growth of *P. acidilactici*

Table 1. Change of free amino acid released from single strain culture at the end of exponential growth. 0 h and 8 h.

Amino acids concentration (mg/l)	*L. salivarius*		*P. acidilactici*		*L. plantarum*	
	0 h	8 h	0 h	8 h	0 h	8 h
Alanine	200 ± 4	215 ± 4.3	200 ± 4	254 ± 5.1	200 ± 4	60.8 ± 1.2
Asparagine	55 ± 0.6	25.3 ± 0.3	55.2 ± 0.6	55.8 ± 0.6	55 ± 0.6	7.8 ± 0.1
Asparatic acid	124.3 ± 1	64.7 ± 0.6	124 ± 1.2	124 ± 1.2	124 ± 1.2	25.7 ± 0.3
Citrulline	45.4 ± 0.5	22.2 ± 0.2	46.4 ± 0.5	21.8 ± 0.2	46 ± 0.5	6.08 ± 0.1
Cysteine	7.48	5.23	7.48	5.73	7.48	0.53
Glutamic acid	428 ± 8.6	358 ± 7.2	428 ± 8.6	565 ± 11	428 ± 8.6	102 ± 2
Glycine	115 ± 2.3	140.7 ± 3	114 ± 2.3	40.6 ± 0.8	119 ± 2.4	38 ± 0.76
Histidine	24.5 ± 0.2	18.6 ± 0.2	23.2 ± 0.2	30.4 ± 0.3	27.9 ± 0.3	6.4 ± 0.1
Lysine	149	124	149	219	149	3.3
Ornithine	5.76 ± 0.1	5.5 ± 0.1	5.53 ± 0.1	6.1 ± 0.1	5.9 ± 0.1	21.6 ± 0.2
Phenylalanine	120.4 ± 2	82 ± 1.6	120 ± 2	144.5 ± 3	120 ± 2	11.4 ± 0.3
Proline	89.2 ± 0.9	80.6 ± 0.8	89.2 ± 0.9	121 ± 1.2	89 ± 0.9	26.4 ± 0.3
Tyrosine	59 ± 1.2	40.7 ± 0.8	59 ± 1.2	67.3 ± 1.3	59 ± 1.2	8.14 ± 0.2
Arginine	272 ± 2.7	77 ± 0.8	272 ± 2.7	427 ± 4.3	272 ± 2.7	72.1 ± 0.7
Isoleucine	104.3 ± 2	77 ± 1.5	104 ± 2.1	142 ± 2.8	105 ± 2.1	26.7 ± 0.5
Leucine	224.8 ± 4	143.6 ± 3	225 ± 5	278 ± 5	224 ± 4	52 ± 1
Methionine	49.4 ± 1	18.6 ± 0.4	50 ± 1	64.7 ± 1.3	52 ± 1	17.8 ± 0.4
Serine	109.6 ± 2	91.1 ± 1.8	109 ± 2.2	135 ± 2.7	110 ± 2.2	28.1 ± 0.6
Threonine	87.9 ± 1.8	66.7 ± 1.3	88 ± 1.8	113.5 ± 2	88 ± 1.8	22.2 ± 0.4
Tryptophan	26.8	14.4	27	43	26	6.6
Valine	155 ± 3.1	126.8 ± 3	153 ± 3	217 ± 4	155 ± 3	40.1 ± 0.8

Data represent the mean values from three independent experiments ($n = 3$) and their standard deviation.

produced many amino acids at levels beyond those needed for the organism's metabolism. In contrast, *P. acidilactici* decreased the medium contents of citrulline, cysteine and glycine, which are essential for the growth of this strain. There was no significant difference with respect to the amount of aspartic acid during both 0 and 8 h, which this strain uses as a growth factor, but not much configuration.

Finally, *L. plantarum* decreased the overall amino acid concentration in the medium, showing particular consumption of alanine, asparagine, aspartic acid, citrulline, cysteine, glutamic acid, glycine, histidine, lysine, phenylalanine, proline, tyrosine, arginine, isoleucine, leucine, methionine, serine, threonine, tryptophan and valine, which are essential for the growth of this strain. *L. plantarum* released ornithine, which is non-essential for its growth.

Comparisons revealed that *P. acidilactici* showed higher growth parameters and efficiently produced a subset of amino acids that complemented the amino acid requirements of *L. salivarius* and *L. plantarum*. Strain *L. salivarius* was superior in cell growth and lactic acid production, whereas *L. plantarum* showed very poor cell growth and lactic acid production (Figure 1).

Effect of mixed-strain culture of *L. salivarius* and *P. acidilactici* on the metabolism of amino acids in MRS medium

During single- and mixed-strain cultures of *L. salivarius*, *P. acidilactici* and *L. plantarum*, we observed the highest cell growth and lactic acid production from mixed-strain cultures of *L. salivarius* and *P. acidilactici.* A previous study showed that mixed cultures of *Lactobacillus casei* and *Lactococcus lactis* showed better lactic acid production and sugar utilization compared to single-strain cultures (Nancib et al., 2009). However, the present study is the first to examine the co-culture of *L. salivarius* and *P. acidilactici* (two strains isolated from pig feces) for enhancement of cell density and lactate production in MRS medium. We speculate that symbiotic associations in the mixed culture were able to overcome (and possibly overcompensate for) the nutritional limitations of the utilized substrate. In an early study, Mills and Thomas (Mills and Thomas, 1981) showed that amino acids and small-molecular-weight proteins may offer very efficient nitrogen sources for the growth of LAB. More recently, Sriphochanart et al. (2011) investigated the effect of amino acid requirements on growth and lactic acid

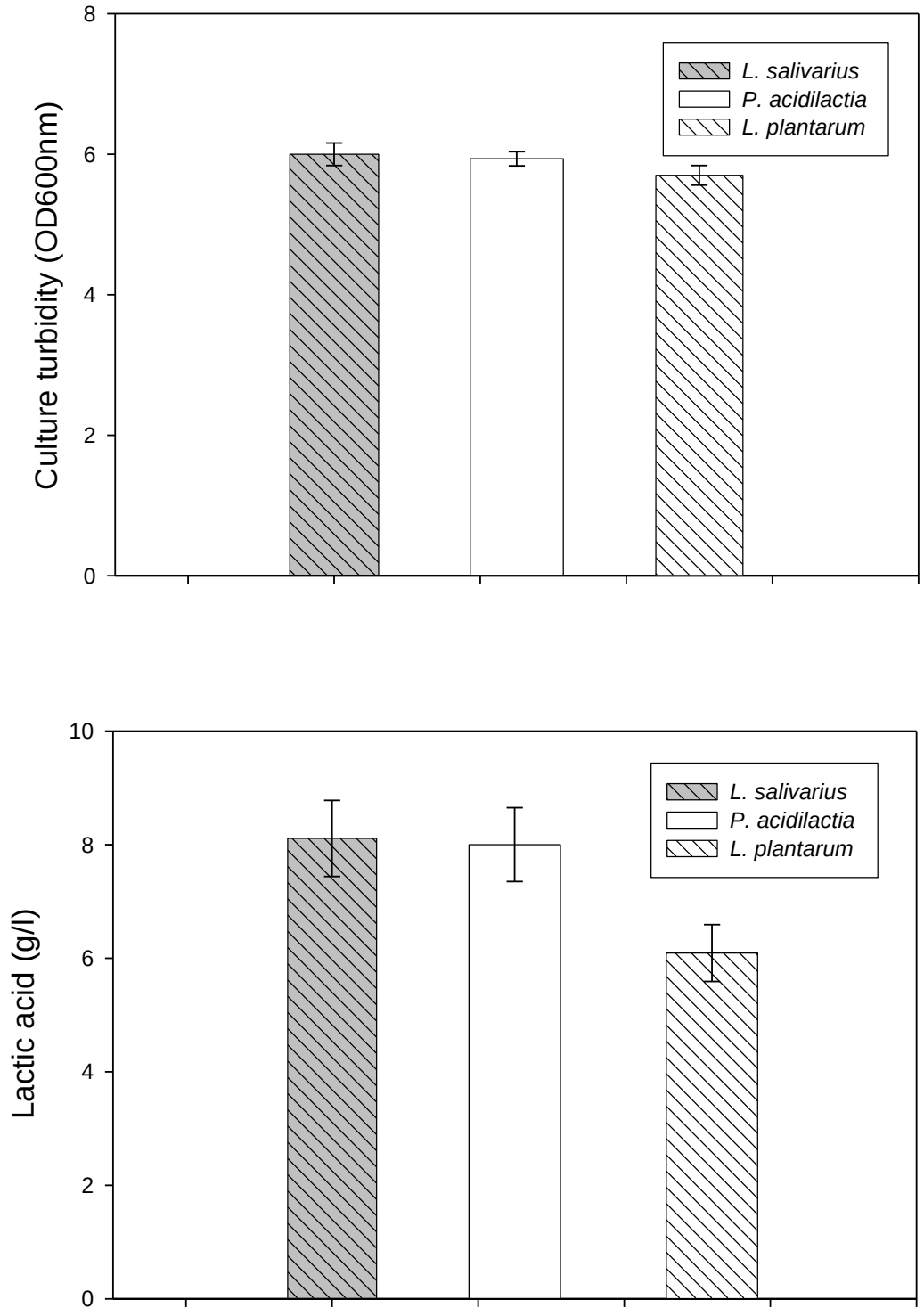

Figure 1. Cell growth and lactic acid production from single-strain cultures. Each number represents the mean ± SD of three replicates.

production in cultures of *P. acidilactici*. In the present study, in an effort to better understand the possible synergetic effects of co-cultured strains, we first examined the free amino acid contents following single-strain cultures of different *Lactobacilli* in MRS medium. We then selected two starter cultures that appeared to overcome each other's nutritional limitations, and showed that the co-culture of *L. salivarius* and *P. acidilactici* increased the

Table 2. Production of free amino acids from mixed cultures grown at optimal inoculation densities at the end of exponential growth (0 h and 8 h).

Amino acids	Ls and Pa (1:1)		Ls and Pa (2:1)		Ls and Pa (5:1)		Ls and Pa (9:1)	
concentration (mg/l)	0 h	8 h	0 h	8 h	0 h	8 h	0 h	8 h
Alanine	200 ± 6	156 ± 5	200 ± 6	232 ± 7	200 ± 6	350 ± 9	200 ± 6	306 ± 9
Asparagine	55 ± 1.7	20.5 ± 1	55 ± 2	29 ± 1	55 ± 2	44.5 ± 1	55 ± 2	33 ± 1
Asparatic acid	124 ± 2	49.8 ± 1	124 ± 2	73 ± 1	124 ± 2	114 ± 2	124 ± 2	95.7 ± 2
Citrulline	45.4	21.2	45.4	18.2	45.4	32.6	45.4	3.12
Cysteine	8 ± 0.1	1.78	8 ± 0.1	4.04	8 ± 0.1	6 ± 0.1	8 ± 0.1	5 ± 0.1
Glutamic acid	428 ± 8	257 ± 7	428 ± 8	388 ± 9	428 ± 8	620 ± 9	428 ± 8	505 ± 9
Glycine	115 ± 2	36.8 ± 1	115 ± 2	173 ± 3	115 ± 2	257 ± 5	115 ± 2	230 ± 4
Histidine	25 ± 0.5	16 ± 0.3	25 ± 0.5	26 ± 1	25 ± 0.5	45 ± 1	25 ± 0.5	33 ± 1
Lysine	149	94	149	129	149	241	149	187.7
Ornithine	5.8 ± 0.1	73 ± 0.7	5.8 ± 0.1	115 ± 1	5.8 ± 0.1	151 ± 2	5.8 ± 0.1	79 ± 0.8
Phenylalanine	120 ± 3	39 ± 1.2	120 ± 3	68 ± 2	120 ± 3	127 ± 4	120 ± 3	113 ± 3
Proline	89 ± 0.9	63 ± 1.3	89 ± 0.9	88 ± 1.8	89 ± 0.9	134 ± 3	89 ± 0.9	120 ± 2
Tyrosine	59 ± 0.6	26 ± 0.3	59 ± 0.6	41 ± 0.4	59 ± 0.6	77 ± 0.8	59 ± 0.6	63 ± 0.6
Arginine	272 ± 3	154 ± 1	272 ± 3	268 ± 2	272 ± 3	193 ± 2	272 ± 3	222 ± 2
Isoleucine	104 ± 1	61.3	104 ± 1	87.3	104 ± 1	143 ± 1	104 ± 1	117 ± 1
Leucine	225 ± 4	112 ± 2	225 ± 4	160 ± 3	225 ± 4	258 ± 5	225 ± 4	217 ± 4
Methionine	49.4 ± 1	20 ± 0.4	49.4 ± 1	21 ± 0.4	49.4 ± 1	17 ± 0.3	49.4 ± 1	668 ± 9
Serine	110 ± 3	65 ± 2	110 ± 3	98 ± 3	110 ± 3	152 ± 4	110 ± 3	131 ± 4
Threonine	88 ± 0.9	53 ± 0.5	88 ± 0.9	76 ± 0.8	88 ± 0.9	141 ± 1	88 ± 0.9	125 ± 1
Tryptophan	27 ± 0.5	8.5 ± 0.2	27 ± 0.5	20 ± 0.4	27 ± 0.5	43 ± 0.9	27 ± 0.5	20 ± 0.4
Valine	155 ± 3	102 ± 2	155 ± 3	135 ± 3	155 ± 3	190 ± 4	155 ± 3	190 ± 4

Data represent the mean values from three independent experiments (*n = 3*) and their standard deviation. Ls, *L. salivarius;* Pa, *P. acidilactici.*

overall cell growth and lactic acid production in our system. In this case, *P. acidilactici* provided essential amino acids (e.g., asparagine, aspartic acid, citrulline, cysteine, glutamic acid, histidine, lysine, ornithine, phenylalanine, proline, tyrosine, arginine, isoleucine, leucine, methionine, serine, threonine, tryptophan and valine) required by *L. salivarius*. In particular, the amounts of asparagine, aspartic acid, citrulline, cysteine and arginine had direct effects on lactic acid production.

In an effort to increase the rate and yield of cell growth and lactic acid production in mixed cultures of *L. salivarius* and *P. acidilactici*, in the hope of showing that this system may be suitable for industrial use, we examined four different *P. acidilactici*: *L. salivarius* inoculation ratios: 1:1, 1:2, 1:5, and 1:9 (calculated based on OD_{600}). Due to differences in the proteolytic rates of the two strains and the possible dominance of one strain over another, different inoculation proportions were tested. As shown (Table 2), incubation of mixed cultures for 8 h increased the supernatant levels of alanine, glutamic acid, glycine, histidine, lysine, ornithine, phenylalanine, proline, tyrosine, isoleucine, leucine, methionine, serine, threonine, tryptophan and valine, which were non-essential for the growth of this mixed culture. In contrast, the mixed cultures consumed asparagine, aspartic acid, citrulline, cysteine and arginine, which are essential for the combined growth of these strains. The overall amino acid concentration increased significantly up to ratio 1:5, and decreased thereafter.

In terms of growth and lactic acid production, the 2:1 ratio showed the highest performance compared to the other inoculation ratios (Figure 2), suggesting that this ratio is most appropriate for our mixed culture. The higher tested ratios of the mixed culture (5:1 and 9:1; *L. salivarius* to *P. acidilactici*) inhibited cell growth, whereas a weaker ratio (2:1) was found to be optimal for cell growth and lactic acid production. We speculate that the stronger ratios (5:1 and 9:1) did not provide sufficient essential amino acids for cell growth and lactic acid production, whereas the weaker ratios (1:1 and 2:1) provided sufficient essential amino acids and probably stimulated the metabolism required for cell growth and lactic acid production.

We hypothesized that stimulation of the proteolytic system in a two-strain mixed culture would increase the release of amino acids that are essential for the growth of both strains. Jini et al. (2011) previously reported that *P. acidilactici* exhibited high proteolytic activity and good antagonism toward bacterial pathogens. In the present

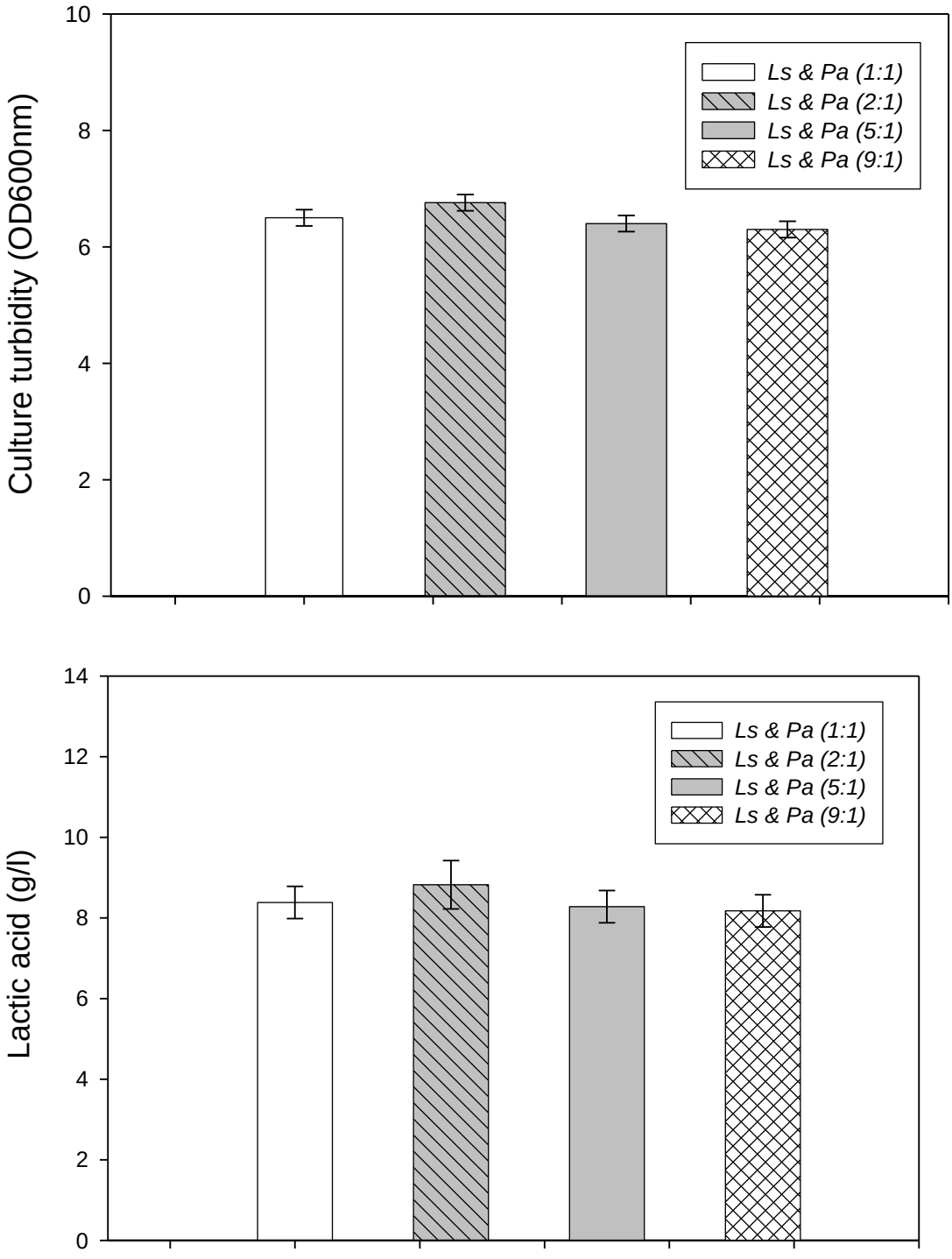

Figure 2. Cell growth and lactic acid production from mixed strain cultures under various inoculation density. Each number represents the mean ± SD of three replicates.

work, proteins were hydrolyzed when the extracellular proteinase produced by *P. acidilactici* accumulated in the medium in pure or mixed cultures of this strain. The proteolytic *P. acidilactici* showed higher growth parameters and efficiently produced a subset of amino acids that largely corresponded to those consumed by *L. salivarius*. We speculate that stimulation of the proteolytic system in a mixed culture of the two would increase the release of amino acids that are essential for the growth of both strains. Hence, we hypothesized that co-culturing

our strains of *L. salivarius* and *P. acidilactici* could enhance the growth of *L. salivarius* and overall lactic acid production.

Conflict of Interests

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

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Full Length Research Paper

Production of ethanol from tuberous plant (sweet potato) using *Saccharomyces cerevisiae* MTCC-170

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The aim of this work was to research a bioprocess for bioethanol production at laboratory scale from raw sweet potato using *Saccharomyces cerevisiae* MTCC-170. In order to obtain maximum conversion of starch into fermentable sugar, optimum parameters for the liquefaction were determined as 104 to 105°C, 0.15% v/w of α-amylase enzyme solution (300 U/ml) and 30 g dry-weight sweet potato mash/100 ml distilled water, respectively with a 74.38% loss in dry weight during the process. For saccharification process, the optimum dose of amyloglucosidase was 0.25% v/w (300 U/ml) with 16.82% glucose production at pH 5.0 and temperature 60°C after 1 h. The fermentation parameters like inoculum size, temperature, pH and different concentrations of nutrients were also determined. The maximum ethanol concentration, that is, 7.95% (v/v) was obtained with 10% inoculum size at pH 6.0 after 48 h. Furthermore, out of the three nitrogen sources (yeast extract, peptone and ammonium sulphate) tested for ethanol production, peptone at a concentration of 1.5 g/L was found to be best (7.93%). From the present study, it may be concluded that sweet potato can be an attractive feedstock for bioethanol production from both the economic stand points and environment friendly.

Key words: Sweet potato starch, ethanol, liquefaction, saccharification, *Saccharomyces cerevisiae* MTCC-170.

INTRODUCTION

Petroleum is the source of about 170 quads of energy out of the total of more than 460 quads used by the world which is far more than derived from other sources (IPCC, 2007). Besides the negative global warming impact of fossil fuels, volatile oil price and political unstable in oil exporting countries resulted in a significant increase in international interest in alternative fuels and led policy makers in the world to issue ambitious goals for substitution of alternative for conventional fuels (Galbe and Zacchi, 2002; Wyman, 2007). Bioethanol made biologically by fermentation from a variety of biomass sources is widely recognized as a unique transportation fuel and original material of various chemical with powerful economic, environmental and strategic attributes.

According to the US Department of Agriculture, recent experiments note that sweet potatoes yield two to

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three times as much fermentable carbohydrate as field corn (USDA, 2008). Sweet potato (*Ipomea batatas*) has been considered as a promising substrate for alcohol fermentation since it has a higher starch yield per unit land cultivated than grains (Duvernay et al., 2013; Lee et al., 2012; Srichuwong et al., 2009; Ziska et al., 2009). Industrial sweet potatoes are not intended for use as a food crop. They are bred to increase its starch content, significantly reducing its attractiveness as a food crop when compared to other conventional food cultivars (visual aspect, color, taste etc.). Therefore, they offer potentially greater fermentable sugar yields from a sweet potato crop for industrial conversion processes. It has been reported that some industrial sweet potatoes breeding lines developed could produce ethanol yields of 4500 to 6500 L/ha compared to 2800 to 3800 L/ha for corn (Duvernay et al., 2013; Ziska et al., 2009). Sweet potato has several agronomic characteristics that determine its wide adaptation to marginal lands such as drought resistant, high multiplication rate and low degeneration of the propagation material, short grow cycle, low illness incidence and plagues, cover the soil rapidly and therefore protect it from the erosive rains and controlling the weed problem (Cao et al., 2011; Duvernay et al., 2013).

Microorganisms meet their energy demand by converting carbon sources to by-products such as: carbon dioxide, lactic acid, ethanol etc. *Saccharomyces cerevisiae, Zymomonas mobilis, Kluyveromyces* spp. and *Schizosaccharomyces pombe* are microorganisms able to convert sugars to ethanol. Various feedstock and chemically defined media can be used for ethanol fermentation. The most commonly used types of feedstock for ethanol production are corn, sugar cane and wheat (Balat et al., 2008). Sugarcane, sugar beets and molasses are feasible for ethanol fermentation and have been used; however, these carbon sources are high value products as food sources (Nalley and Hudson, 2003; USDA, 2006). In order to meet the low cost requirement, lignocellulosic biomass is another option for ethanol fermentation. However, lignocellulosic biomass is complex and requires expensive pre-treatments. Currently, sweet potatoes are alternative feedstock for ethanol production. Sweet potato like other starchy root crops is a cheaper substrate and can therefore serve as raw material for fermentative production of commodity chemicals. Cultivated in more than 100 countries, sweet potato ranks third of the world root and tuber crops production after potato and cassava (FAO stat, 2010).

The sweet potato (Pusa Lal) used in this work was identified as a sustainable crop for bioethanol production based on both its favourable energy balance and the net greenhouse gas (GHG) emission reduction, evaluated on a life cycle analysis conducted for local conditions in Uruguay (Carrasco-Letelier et al., 2013). It was developed as culture for bioenergy purposes on the basis of its high starch yields. This sweet potato variety has significantly reduced its attractiveness as a food crop when compared to other conventional food cultivars. The main objective of this work was to develop an economical bioprocess technology to produce bioethanol from raw sweet potato at laboratory scales and determined the effect of fermentation temperature, inoculum sizes, pH and effect of different nutrients on fermentation parameters.

MATERIALS AND METHODS

Characterization of raw material: Sweet potato

Raw fresh sweet potato (Pusa Lal) harvested in February 2009 was procured from Ch. Charan Singh Haryana Agriculture University, Hisar and was stored at room temperature (at about 20°C for 30 days). Thoroughly washed peeled sweet potato (1.0 kg) were dried overnight at 70°C and grounded to fine powder. The carbohydrate composition of sweet potato flour was determined by 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). Protein concentration was determined by the Kjeldahl method. Ashes were quantified by gravimetric analysis after burning samples at 550°C for 5 h. Moisture content was determined by gravimetric analysis after drying at 105°C to constant weight. The C, H, N was analyzed by standard methods (AOAC, 1990).

Enzyme for liquefaction and saccharification

Commercial α-amylase (Specific activity 300 DUN U/ml) and amyloglucosidase (Specific activity 400 GA U/ml) were obtained from Sigma-Aldrich Pvt. Ltd., India.

Preparation of sweet potato flour slurry

Slurries of various concentrations (10, 15, 20, 25 and 30% w/v) of sweet potato flour starch was prepared in water and treated with liquefying enzyme (0.15% v/w) at 104 to 105°C for 60 min in an autoclave. The slurry prepared by mixing 25 g flour in 100 ml water (1:4) being homogenous, loose, easy to handle was used for further experiments. Liquefaction of sweet potato flour (100 ml slurry) was carried out at 104 to 105°C in an autoclave using varying concentration of enzyme (0.05 to 0.20% v/w) for different time intervals (10 to 240 min). The progress of liquefaction was monitored by employing starch-iodine (1.0 g of iodine and 2.0 g KI in 100 ml water) reaction. Saccharification of liquefied starch was carried out at 60°C for different time intervals using varying concentration (0.05 to 0.45% v/w) of amyloglucosidase. The reaction was monitored by the yield of total reducing sugars estimated by dinitrosalicylic acid method (Miller, 1959).

Yeast strain

A fast fermenting strain of *S. cerevisiae* MTCC-170 was obtained from Microbial Type Culture Collection, Chandigarh (India) and maintained on yeast extract peptone dextrose (YEPD) agar medium containing yeast extract (1%), peptone (2%), dextrose (2%) and agar (2%). Dextrose inoculum medium used for inoculum preparation contained dextrose (6%), peptone (0.5%) and yeast extract (0.5%). Yeast cells pregrown in inoculum medium for 18 h under shaking condition (100 rpm) was used directly as inoculum at 10% (v/v).

Table 1. Composition of starchy raw materials.

Raw material	Source	Chemical composition % (w/w)					
		Starch content		Nitrogen content	Protein content	Phosphorus content	Ash content
		Acid hydrolysis	Enzymatic hydrolysis				
Sweet potato (Pusa Lal)	CCS HAU, Hisar	69.26	70.34	0.75	4.50	0.56	4.10

Optimization of fermentation conditions

Effect of inoculum concentration

The hydrolysate was inoculated with different concentrations of inoculums; that is, 5, 10, 15 and 20% (v/v) and kept for fermentation at 35°C for 48 h.

Effect of temperature

The hydrolysate inoculated with the best combination of nutrients and fermentation was carried out at various temperatures viz. 25, 30, 35 and 40°C. Ethanol content in fermented samples was estimated after 48 h of incubation.

Effect of pH

The pH of hydrolysate was adjusted to different levels and was fermented after supplementation with the best combination of nutrients after inoculating with 10% inoculum (v/v). The fermentation was carried out at 35°C for 48 h.

Effect of nutrient concentration

To 100 ml hydrolysate, different nutrients like ammonium sulphate (0.3%), yeast extract (0.5%) and peptone (0.5%) was added in their single and double concentration. The flasks were inoculated with 10% yeast cells (v/v). The fermentation was carried out at 35°C for 48 h.

Analytical methods

Estimation of reducing sugars

The DNS method given by Miller (1959) was used to estimate reducing sugars of the samples.

Ethanol determination

Ethanol concentration was determined by the method given by Caputi et al. (1968). All reagents were of analytical grade. Concentrations were calculated by means of standard curves relating individual concentration to peak area. Every experiment was conducted in triplicate.

Calculations

The maximum theoretical yield of ethanol from sugar was calculated according to the stoichiometric relation given in Equation (1), that is, 100 g of hexose produce 51.1 g of ethanol and 48.9 g of CO_2. Ethanol yields over total initial sugars (Y_1) and average ethanol productivity rate (Y_2) were calculated according to Equations (2) and (3) as given by Zhang et al. (2011).

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \quad (1)$$

$$Y_1 = \frac{\text{ethanol produced in fermentation}}{\text{ethanol produced in theoretical}} \times 100 \quad (2)$$

$$Y_2 = \text{final ethanol concentration/fermentation time} \quad (3)$$

Statistical analysis

All experiments were carried out in a completely randomized design. The results were subjected to analysis of variance (one-way ANOVA), and the treatment means were compared using the least significant difference (LSD) values at a significance level of $P < 0.05$.

RESULTS AND DISCUSSION

Composition of sweet potato

Carbohydrate composition of sweet potato flour was analyzed by hydrolyzing the sweet potato flour by 54% concentrated perchloric acid at high temperature of 100°C for 2 h. Total glucose derived from starch, cellulose and soluble portion occupied approximately 91% of dry matter.

The total carbohydrates concentration of sweet potato tuber was 76.34% (v/v) and contained 63.30% (v/v) of starch, 70.34% (v/v) of glucose. Nitrogen contents, ashes, protein contents and phosphorus contents of sweet potato mash were approximately 0.75% (w/v), 4.10% (w/v), 4.50% (v/v) and 0.56% (v/v), respectively (Table 1).

Optimization of condition for liquefaction process

The optimum combination of temperature, dose of enzyme (α-amylase) and amount of sweet potato flour slurry was determined as 104 to 105°C, 0.10% v/w of α-amylase enzyme solution (300 U/ml) and 30 g dry-weight sweet potato mash/100 ml distilled water, respectively

Table 2. Summary of liquefaction.

Raw material	Slurry % (w/v)	Enzymes % (v/w)	pH	Temperature (°C)	Time (h)	Ca^{++} (mM)	K^{+} (mM)
Sweet potato	25	0.10	6.2-7.0	104-105	1	0.36	0.30
Pusa Lal	30	0.10	6.2-7.0	104-105	1	0.72	0.30

Table 3. Summary of saccharification.

Raw material	Slurry %(w/v)	Enzymes % (v/w)	pH	Temperature (°C)	Time (h)	Sugar production %(w/v)
Sweet potato	25	0.25	5.0	60	1	14.58
Pusa Lal	30	0.25	5.0	60	1	16.62

with a 68.86% loss in dry weight during the process (Table 2). Kumar et al. (2013) used different concentrations (15 to 45% dry weight/volume) of potato powder for maximum liquefaction, which was carried out using steam under pressure (0.3 to 0.4 lbs, 104 to 105^{o}C) to liquefy the slurry in 1 h. Slurry having 25 and 30% substrate concentration was found to be the best. Alpha-amylase dose optimized for the liquefaction process was 0.15% v/w.

Optimization of saccharification

Dose of enzyme, temperature and saccharification time were optimized for the saccharification process. The optimum dose of amyloglucosidase was 0.25% v/w (300 U/ml) with 16.62 g/100ml glucose production after 1h at 60°C for sweet potato (Table 3). Kumar et al. (2013) observed that optimum temperature for saccharification was found to be 60°C, pH was 5.0 and dose of amyloglucosidase was 0.35% (v/w) when potato was used as a substrate.

Optimization of fermentation conditions

Optimization of inoculum size for ethanol production

In the present study, initial total carbohydrate concentration was 168.1 g/L. In order to determine the economic inoculums size, the inoculums sizes ranging from 5 to 20% were tested for ethanol production using SSF of sweet potato flour with *S. cerevisiae* MTCC-170. As shown in Figure 1, there was significant difference among the inoculum sizes (5, 10, 15 and 20%) tested w.r.t. kinetic parameters in ethanol production. The maximum ethanol concentration (6.73% v/v), sugar utilization (78.19%) and ethanol yield (74.70%) were obtained with an initial inoculum of 10%, which is economic and environment friendly. The shortening of fermentation time with the raise in the inoculum size was due to the fast cell growth within the reactor and most of the substrate was immediately converted to ethanol. According to the study of Fadel (2000), the maximum alcohol production (12.9%) was obtained when inoculated with 10% culture of *S. cerevisiae.* Similarly, Afifi et al. (2011) also observed maximum ethanol production from industrial solid potato wastes when inoculated with 10% (v/w) inoculums size of *S. cerevisiae.* Different inoculum sizes (2, 4, 6, 8 and 10% v/v) were tested for a period of 24 h and observed that the maximum ethanol concentration that is, 8.8% was obtained at 10% inoculum size (Neelakandan and Usharan, 2009). Turhan et al. (2008) reported that maximum ethanol concentration, ethanol productivity and ethanol yield were 42.90 g/L, 3.7 g/L/h and 45%, respectively, obtained with an initial inoculum of 3% when carob extract used as a substrate by using *S. cerevisiae*.

Effect of temperature on ethanol production

Effect of temperature on growth and ethanol production of *S. cerevisiae* MTCC- 170 was also studied. Different temperatures (30, 35 and 40°C) were tested to check the thermal tolerance of *S. cerevisiae* MTCC-170 in sweet potato flour media. From the present study, it was observed that 35°C was the most appropriate temperature for yeast growth. Production of ethanol by *S. cerevisiae* MTCC-170 was also favoured by this temperature and reached its maximum (7.99% v/v) after 48 h. At 45°C, ethanol production was reduced to 2.26% v/v. Both low and high temperature (30 and 40°C) had detrimental effect on ethanol production and reduced it to 7.50 and 4.30% v/v, respectively (Figure 2). Thermostability of a yeast strain is more likely genetically controlled.

Variation in thermal requirements for biomass and ethanol production stimulates the suggestion that enzymes involved in ethanol fermentation, vary in their thermal optima than those that are involved in biomass synthesis. Hashem and Darwish (2010) observed that production of ethanol by *S. cerevisiae* y-1646 was

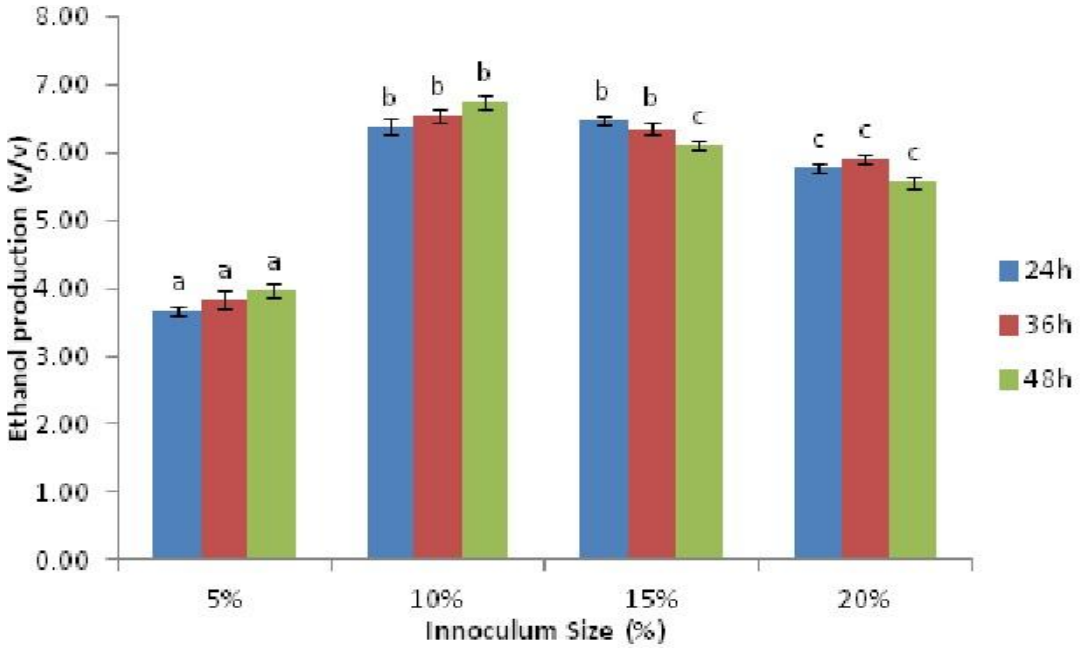

Figure 1. Effect of inoculums size on ethanol production from supplemented sweet potato flour hydrolysate. Values followed by the same letter are not significant at 5% level.

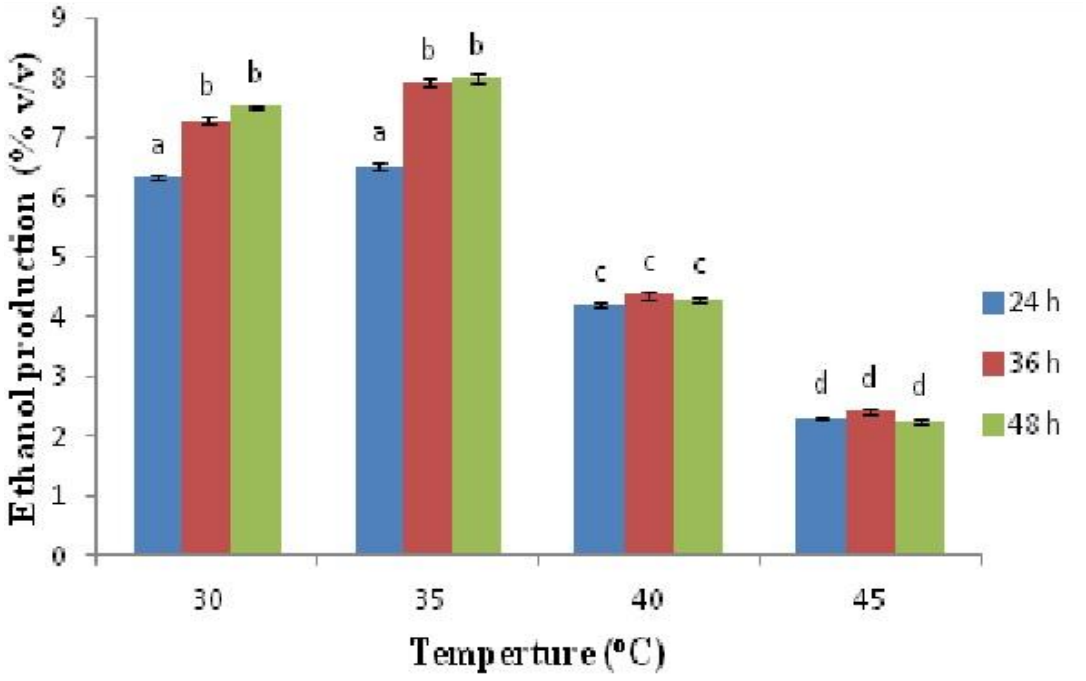

Figure 2. Effect of temperature on ethanol production from supplemented sweet potato flour hydrolysate. Values followed by the same letter are not significant at 5% level.

favored at 35°C temperature and reached its maximum value (5.29 g/L) after 36 h. At 37°C, ethanol production was reduced to 4.38 g/l. Rivera et al. (2006) considered the temperature as the variable to evaluate the optimum expected parameter of ethanol fermentation. Based on experimental data, maximum ethanol production is achieved at 28 to 31°C.

Rani et al. (2010) observed that maximum ethanol

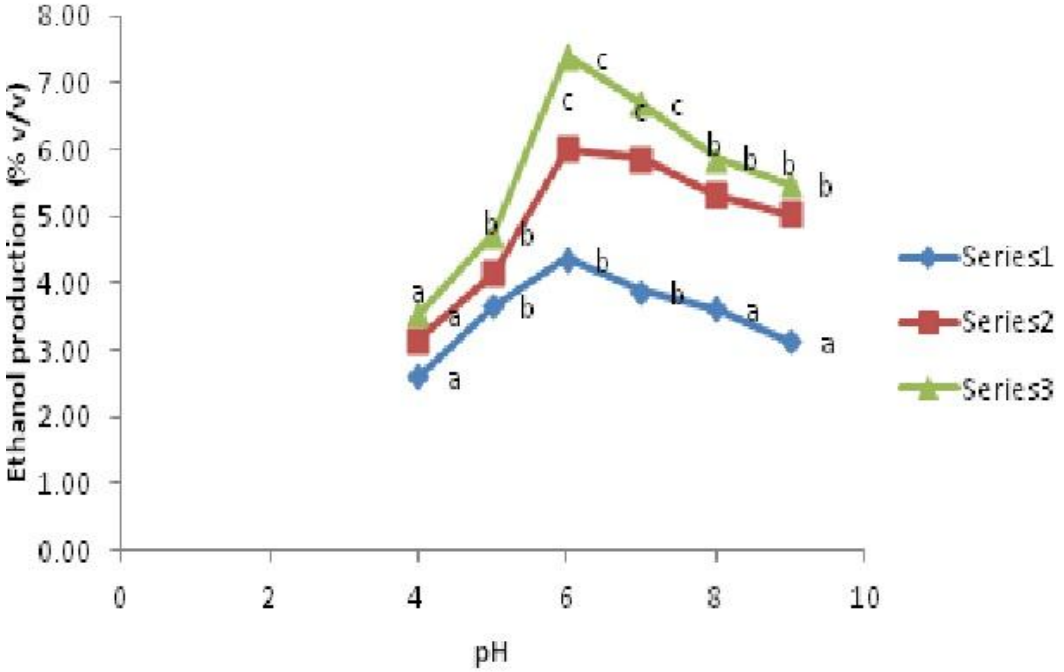

Figure 3. Effect of pH on ethanol production from supplemented sweet potato flour hydrolysate. Values followed by the same letter are not significant at 5% level. Series 1, 2 and 3 represent 24, 36 and 48 h fermentation, respectively.

content of 56.8 g/L was recorded after 48 h of fermentation at 30°C. However at temperatures 35, 37 and 40°C, the corresponding values were 53.6, 50.0 and 46.0 g/L, respectively showing a decline with increase in temperature of fermentation. Duhan et al. (2013) observed bio-ethanol production increases with increase in temperature and reaches its maximum value at 35°C. Asli (2010) observed best ethanol production rate at 32°C temperature. Osman et al. (2011) obtained maximum ethanol production and biomass from sugar cane bagasse at 28 to 30°C.

Effect of pH on ethanol production

The pH is one of the most important factors for any fermentation process and depends upon microorganisms because each microorganism possesses pH range for its growth and activity. Increase or decrease in pH on either side of the optimum value resulted in decrease in growth and activity of microorganisms. Ethanol fermentation was evaluated at different pH profiles (ranges) to determine the effect of pH on ethanol production. As shown in Figure 3, the ethanol concentration was increased from pH 4.0 to 6.0 and decreased marginally above this value. The maximum ethanol concentration 7.41% was obtained when culture (*S. cerevisiae* MTCC-170) was grown at pH 6.0. Fadel (2000) reported that high ethanol production was obtained by using initial pH 5.0 to 6.0. Graves et al. (2006) observed that no ethanol production exists lower than pH 4.0. Turhan et al. (2008) also reported that maximum ethanol yield, growth rate and biomass concentration were obtained at pH 5.5 on carob as a medium for ethanol production. Osman et al. (2011) tested wide initial pH range and found that at pH 3.0, no growth was observed and no ethanol was produced, while pH 6.0 was the optimum for both biomass and ethanol production. Mohanty et al. (2009) reported that pH 6.0 was optimum for bioethanol production from mahula (*Madhuca latifolia* L.) flowers by solid-state fermentation. Togarepi et al. (2012) also obtained maximum ethanol production at pH 6.0 when *Ziziphus mauritiana* fruit pulp was used as a substrate. Afifi et al. (2011) optimized pH; that is, 3.5 for maximum ethanol production from industrial solid potato wastes. Kundiyana et al. (2010) studied the fermentation of sweet sorghum juice at different pH levels and observed that the highest ethanol yield could be obtained at a pH of 4.3.

Effect of nutrients on ethanol production

Effect of ammonium sulfate (nitrogen source) on ethanol production

The effects of three nutrients (ammonium sulfate, dipotassium hydrogen phosphate and yeast extract) on the ethanol yield from sweet potato were investigated.

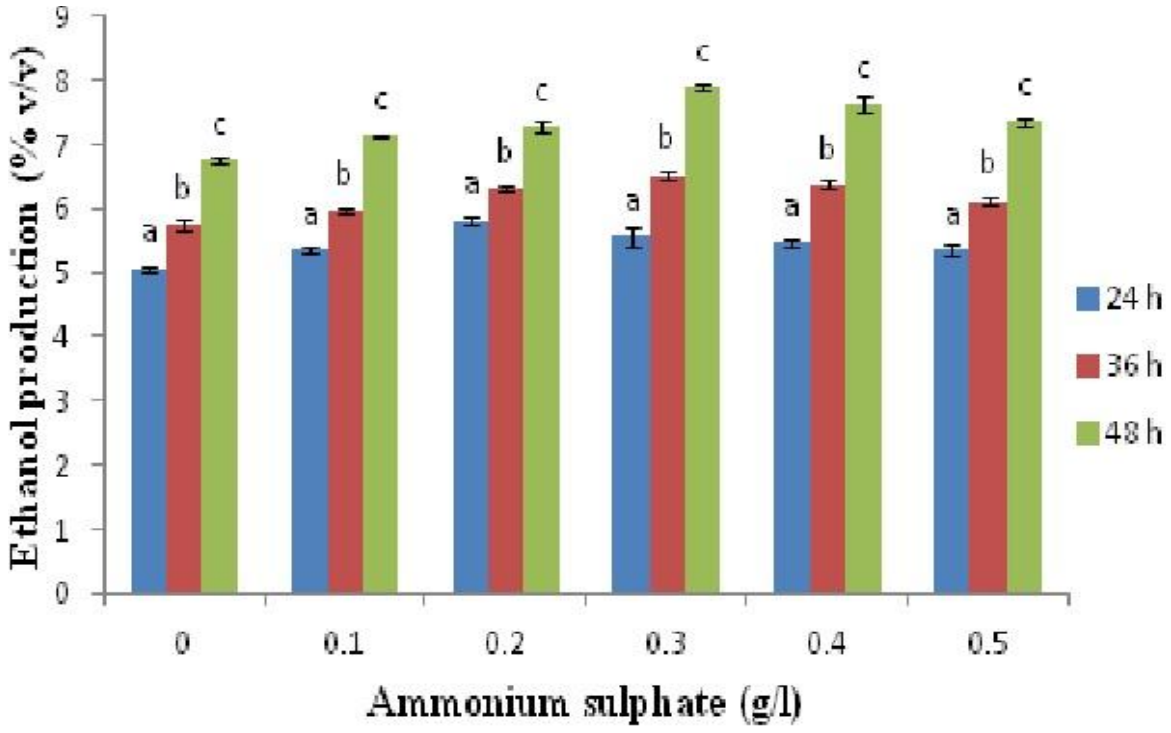

Figure 4. Effect of ammonium sulphate on ethanol production from supplemented sweet potato flour hydrolysate. Values followed by the same letter are not significant at 5% level.

Effect of ammonium sulphate as a nitrogen source was studied by varying its concentration between 1.0 to 5.0 g/L keeping rest of the parameters at their optimal conditions. Data from the Figure 4 shows that as the concentration of ammonium sulphate increased from 1 to 3 g/L, ethanol production also increased from 6.98 to 7.28% for *S. cerevisiae* MTCC-170, above this concentration, ethanol production decreases when sweet potato was used as substrates. Beltran et al. (2007) studied the effect of ammonium sulphate with different concentrations ranging from 0.01 to 0.09 g/L and observed that maximum production was obtained at 0.06 g/L concentration of ammonium sulphate.

Similarly, Amutha and Gunashekaran (2000) also observed that ethanol yield increase from 44.2 and 54.9 g/L, by supplementation of liquefied cassava starch with ammonium sulphate (1 g/L). Srichuwong et al. (2009) studied the saccharification simultaneous fermentation of very high gravity (VHG) potato mash for the production of ethanol and obtained 2.0 to 2.5% more ethanol concentration as compared to control when supplemented with ammonium sulphate. Anupama et al. (2010) obtained optimum ethanol yield of 5.6% with 3 g/L concentration of ammonium sulfate used as a nitrogen source. Slight increase in growth and ethanol production by *S. cerevisiae* y-1646 was observed after addition of NH_4NO_3 (4 g/L) as a source of nitrogen (Hashem and Darwish, 2010).

Effect of yeast extract on ethanol production

Yeast extract proved to be very efficient for increasing fermentation rate, but yeast extract is an expensive additive, which should at least be added in smallest possible amounts in order to make the process economical viable. Effect of yeast extract was studied by varying its concentration from 1.0 to 3.0 g/L, keeping rest of the parameters at their optimal conditions. Data from the Figure 5 shows that as the concentration of yeast extract increased from 1.0 to 2.0 g/L, ethanol production also increased from 6.55 to 7.11% with *S. cerevisiae*, above this concentration, ethanol production was decreased when sweet potato was used as substrates. Likewise, Nuanpeng et al. (2012) studied the effect of yeast extract concentrations on sugar consumption, ethanol production and yeast cell viability during very high gravity batch fermentation of *S. cerevisiae* NP 01 from sweet sorghum juice and observed the highest ethanol concentration in the EP medium containing 9.0 g/L of yeast extract. Thomas and Ingledew (1992) observed that 1% yeast extract supplementation stimulate VHG fermentation of wheat mash to yield 21% (v/v) of ethanol within 4 days. Similarly, Duhan et al. (2013) showed that maximum ethanol production that is, 7.11% was obtained at 2.0 g/L yeast extract for *S. cerevisiae* when potato (Kufri Bahar) was used as substrates.

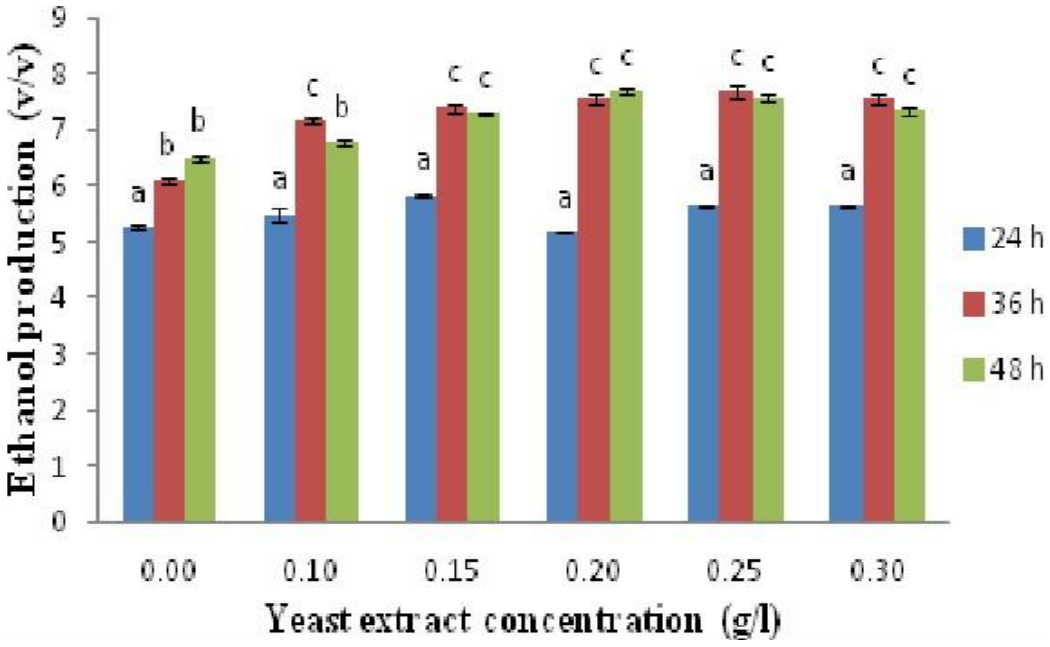

Figure 5. Effect of yeast extract on ethanol production from supplemented sweet potato flour hydrolysate. Values followed by the same letter are not significant at 5% level.

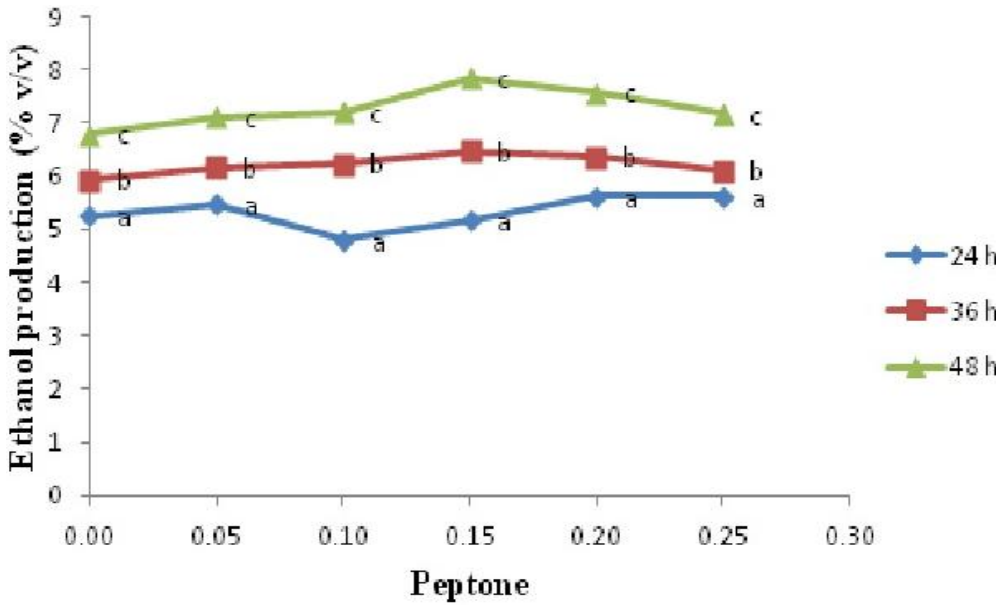

Figure 6. Effect of peptone on ethanol production from supplemented sweet potato flour hydrolysate. Values followed by the same letter are not significant at 5% level.

Effect of peptone on ethanol production

To study the effect of peptone on ethanol production on various concentrations ranging from 0.5 to 2.5 g/L were used. Data in the Figure 6 shows that as the concentration of peptone increased from 0.5 to 1.5 g/L, ethanol production also increases from 5.17 to 7.86% with *S. cerevisiae* and above 1.5 g/L concentration ethanol production was decreased when sweet potato was used as substrate. Wang et al. (2007) studied that 1.5% (w/v) peptone in the medium increased the final ethanol titre from 14.2% (v/v) to 17% (v/v) in 48 h.

Addition of peptone at a concentration of 1% reported to play a very important role in increasing the ethanol yield and the rate of fermentation (Fundora et al., 2000). Dake et al. (2010) obtained maximum ethanol concentration at 0.5% (w/v) of peptone concentration.

Conclusion

Sweet potato flour prepared by oven drying, mashing and grinding was used for ethanol fermentation by *S. cerevisiae* MTCC-170. According to the results in terms of liquefaction, the process conducted at 105°C using 30 g flour of sweet potato and 0.10% v/w α-amylase for 1 h was found to be the most suitable, considering higher liquefaction yield, and when saccharified with gluco-amylase (20.5 GA U/g starch) at 60°C for 2 h the maximum amount of fermentable sugar was released from sweet potato flour that is, 16.84 g/100 ml. In the present study, experimental conditions were tested for liquefaction and saccharification, revealing the higher performance of α-amylase and amyloglucosidase. The addition of nitrogen sources in the fermentable medium increase the ethanol production. The other conditions were also standardized as temperature 35°C, pH 6.0, fermentation medium containing 168.1 g/L reducing sugars supplemented with 1% ammonium sulphate as nitrogen source, inoculum size of 10% of 24 h yeast culture (0.01 at 600 nm) and shaking rate 120 rpm for maximum ethanol production. Finally, 88.1 g/L ethanol was detected under these optimum conditions by batch fermentation. According to the results, it could be concluded that sweet potato is an attractive feedstock for the bioethanol production, since it provided the necessary nutrient element and the appropriate hydrogen balance for the fermentation.

Conflict of Interests

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

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Full Length Research Paper

Immuno-histochemical localization of cholesterol binding proteins in *Schistocerca gregaria* (Forskal)

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This manuscript aims to investigate immunocytochemical localization of cholesterol binding proteins (CBPs) in semi-thin sections of midgut of *Schistocerca gregaria* (Forskal). For this purpose, polyclonal antibody specific to CBPs were raised in albino mice and used in immuno-fluorescence and immuno-blotting to determine the cellular location of CBPs. Midgut tissue sections were incubated with pAbs anti-cholesterol binding protein (primary antibody) and finally associate them with HRP conjugated anti-rabbit immunoglobulin (secondary antibody). Semi thin tissue sections of midgut portion were stained with hematoxylin and eosin for establishing general morphology of epithelial cells in control sections. Positive control tissue sections were stained with Sudan Black-B for microscopic visualization of cholesterol binding sites. Further, cholesterol association in tissue sections was confirmed by using tetramethylrhodamine isothiocyanate (TRITC) labeled florescent antibodies and immuno-blotting of CBPs. Finally, CBPs or cholesterol-carrying proteins were detected intracellularly in midgut epithelial/ microvillus cells named as CBP+. Zymogene like dense granules localized were found scattered throughout the apical portion of microvillus cells. Further, presence of these CBPS was confirmed by SDS-PAGE gel electrophoresis and immuno-blotting. In treatments, dietary cholesterol was found to be internalized bound to complexed with CBPs before absorption. Further, same protein was also localized in other tissues like fat body, testis, and ovary of male and female insects of *S. gregaria.* However, present study done on immuno-cytochemical localization of cholesterol binding proteins in microvillus cells confirms that CBPS are main careers of cholesterol. These proteins also assist in transport of cholesterol to the various organs after its subsequent absorption.

Key words: Cholesterol binding proteins (CBPs), cholesterol, lipoprotein, midgut, immunocytochemical localization, *S. gregaria* (Forskal).

INTRODUCTION

Insects are unable to synthesize sterols de novo and rely on it exclusively on the exogenous source from diet (Laser and Clayton, 1966; Agarwal, 1970; Svoboda and Weirich, 1995) to fulfill their nutritional requirements (Lipke and Frankel, 1956). Sterols are indispensable components of animal cells, which conjugate with proteins to form lipoproteins. In many animals and plants, cholesterol is biosynthesized from smaller molecules or acetates but in insects, this pathway is totally absent (Gilbert, 1967; Clayton et al., 1964). Mostly phytophagous insects' intake plant sterols in the natural diet and converts them into zoosterols and metabolizes them (Thomas and Gilbert, 1968). After the intake through diet, sterols are absorbed inside insect midgut (Joshi and Agrawal, 1977), in which some carrier proteins are involved (Gong et al., 2006). These proteins also serve as carrier vehicles of sterols (Nemeez and Schroeder, 1991; Mayer et al., 1985) and transport different types of lipids

mainly diacylglycerol (Chino et al., 1977) phospholipids (Klucken et al., 2000), cholesterol (Upadhyay et al., 2002) and hydrocarbons to various sites inside insect body (Abbey et al., 1985; Yun et al., 2002). These lipid carrier proteins are synthesized in fat body and play an important role in absorption and transport of different types of lipids such as diacylglycerol, cholesterol and phospholipids. These proteins also play very important role in transfer or exchange of lipids (Vahouny et al., 1985) across/between the membranes (Tam et al., 2006). After binding to surface receptors (Radhakrishnan et al., 2004; Wang et al., 2006) these protein assist in intracellular distribution of sterols mainly cholesterol (Upadhyay and Agarwal, 2007; Bass, 1988: Haunerland et al., 1992) and maintain cholesterol homeostasis inside cells (Brown and Goldstein, 1986). Similar few fatty acid binding proteins (M- FABPs) are purified also from different mammalian tissues (Ockner and Manning, 1982), which carry fatty acids to mitochondria for subsequent β-oxidation in flight muscles (Haunerland and Chisholm, 1990; Simons and Ikonen, 2000).

Besides lipoproteins, vitellogenins also transport lipids to developing insect oocytes (Atella et al., 2006). These proteins are major components of mature insect eggs and are considered as precursors of lipoproteins, and play a dual role in transport of lipids from fat body to ovaries. These are synthesized in the fat body and secreted into the hemolymph from where these are transferred to maturing follicles (Osir et al., 1986). These sterol carrier proteins have been identified and immunocytochemically localized in, liver cells (Pandak et al., 2006), rat brain (Horton and Shimomura 1999; Kim and Ong, 2009) and in mosquitoes (Komnick and Giesa, 1994; Lan and Massey, 2004; Schroeder et al., 2000). In addition, intracellular localization of insect sterol carrier protein-2 was successfully done (Seedorf et al., 2000). The sterol carrier proteins have been previously purified from insects but its cellular localization has not yet been established. Hence in the present investigation, immunecytochemical localization of CBPs was done in semi-thin sections of midgut of *S. gregaria*.

MATERIALS AND METHODS

Insects

Locusts also known as *S. gregaria* (Forskal) were cultured in the laboratory by feeding on natural diets. Animal culture was maintained in the laboratory conditions at 27 ± 3°C, 40 to 60% humidity and a photoperiod of 12L: 12D. Experimental animals were made disease free by treating them with solvents and antibiotic substances (Streptomycin20 µg/ml and fungizone 20 IU/ml).

Feeding the animals

Adult males and female of insects *S. gregaria* (in 10 to 15 in num-0ber) were placed in separate cages (18"×14"×16' unit') at room temperature. For treatment, insects were fed on cholesterol solution (300 µg/ml, Merck India) coated on green leaves. Unfed insects were excluded from the observations.

Preparation of homogenate

Midgut tissues (500 mg) were obtained from *S. gregaria* according to Thomas (1984) and were homogenized in a glass-glass homogenizer in 5 ml of Tris-HCl buffer (5 mM, pH7.8). Homogenate was centrifuged at 12 000 rpm for 30 min at 4°C in cold centrifuge (Remi) and supernatant was taken out for estimation of protein using Lowry's method (Lowry et al., 1951). From the supernatant, three aliquots of 10 µL each were used for estimation of proteins**.**

Purification and characterization of CBPs

CBPs were purified by gel filtration chromatography (column dimensions). For this, regular fractions of midgut homogenate of locusts were eluted (24 ml/h) and collected according to method of Spier (Spier, 1982). Flow rate was maintained between 20 to 24 ml per h by using a continuous buffer supply in a cold room. Eluted fractions were collected manually at a fixed interval of time at a constant flow rate. More than 130 fractions were eluted.

Protein estimation in eluted fractions

Protein contents in the eluted fractions were estimated using Lowry's method (Lowry et al., 1951) and the values were plotted to show the presence of proteins on graph. Absorbance was taken at 640 nm in each fraction to get the elution pattern of CBPs. Besides these, eluted fractions were also evaluated at a wavelength of 260 and 280 nm.

Molecular weight estimation of CBPs

Proteins of known molecular weights were passed through a gel filtration column at the same speed at which samples were eluted. The elution volumes of unknown proteins were compared with the log values on the x-axis for estimation of molecular weight.

Production and purification of Polyclonal antibodies

Young albino mice (*Mus musculus*) weighing around 30 ± 5 gm were used for immunization. Disease free animals were purchased from animal supplying agency and kept for one week in laboratory for acclimatization.

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Abbreviations: PBS, Phosphate buffer saline; **SDS-PAGE,** sodium dodecyl sulphate polyacrylamide gel electrophoresis; **pAbs,** polyclonal antibodies; **CBPs,** cholesterol binding proteins; **mv,** microvillus cells; **lm,** lumen; **ovf,** ovarian follicles; **tsf,** testicular follicles.

Immunization

Polyclonal antibodies (pAbs) directed against CBPs were raised in albino mice. Mice were injected with complete immunogen subcutaneously and intra-peritoneally. Only one injection of 0.30 mg of purified CBPs was given to each mouse and booster injection was given after 7th day of primary immunization. The first injection of immunogen was prepared by emulsifying purified CBPs with Complete Freund's adjuvant while the last two boosters contained incomplete adjuvant.

Anti-serum was separated by centrifugation. pAbs were purified from sera (10 ml) by precipitation with 50% of saturated ammonium sulphate following octanoic acid precipitation. In one volume of antiserum, two volume of sodium acetate buffer (60 mM, pH 4.0) were added at room temperature. Now 0.68 g n-octanoic acid were added drop wise per 10 ml of original antiserum, mixed thoroughly for 30 min and centrifuged at 1000 × *g*. After ammonium sulphate precipitation, pAbs were dialyzed against Tris-HCl buffer (Tris 100mM, NaCl 50 mM, pH 7.8). The serum was loaded on a Sep-Pak column (2.6 × 10 cm equilibrated with Tris HCl buffer (pH 7.8).

For determination of relative concentration of antibodies and antigens, and to find confirmation of antigen and antibody interaction, Ouchterlony method was followed. For testing, four peripheral and one central well were cast in a molten agar gel glass slide (8 × 6 cm) by holes made. In the central well, 100 µL of polyclonal antibody (100 µg/ml) was filled up (140 µg/ml) while in peripheral wells, 100 µL of purified CBP (100 µg/ml) was used. It was kept inside a humidified box in cold (4°C) for double diffusion over night.

Electrophoretic isolation of cholesterol binding proteins

The eluted fractions containing Caps were pooled and lyophilized to a desired concentration of protein. Proteins were separated on native and SDS polyacralymide gel electrophoresis by using method of Laemli (1970). Gels containing CBPs separated on SDS-PAGE were fixed in methanol: glacial acetic acid solution and stained with Comassie Brilliant Blue R 250. Gels were immersed in at least 5 volumes of the staining and placed on a slowly rotating plateform for 4 to 8 h at room temperature. The over stained gels were de-stained in 30% methanol, 10% glacial acetic acid, and 60% water. The gels were photographed by a photo-densitometer camera and stored in water containing 20% glycerol in a glass tank. The specificity of pAbs anti-CBPs was estimated by protein blotting. For it, proteins were separated on SDS-PAGE and transferred to nitrocellulose membrane. Membranes were rinsed three times with PBS (phosphate buffer saline, 10 mM phosphate, NaCl 150 mM, pH 7.2) then saturated with 3% skimmed milk in PBS for 1 h at room temperature. Thereafter, membranes were incubated in pAbs anti-CBP diluted at 1:1000 with PBS containing 0.05% Tween-20. Membranes were further washed twice in fresh PBS/Tween-20 and incubated for 1 h at room temperature with a 1:20 000 dilution of alkaline phosphatase-conjugated anti-rabbit immunoglobulin (Banglo-Genei). After washing as above, membranes were incubated with substrate solution of phosphatase containing 0.3 mg/ml of nitro-blue tetrazolium chloride (Banglo-Genei), 0.2 mg/ml of 5-bromo-4chloro-3indodylphosphate (Banglo-Genei) and 0.2 mg/ml of $MgCl_2$ to reveal the specific immuno-reactivity.

Immuno-cytochemical localization of CBPs

Insects *S. gregaria* were kept for 24 h to feed on water swabs. These were provided pure crystalline cholesterol after dissolving it in absolute alcohol. Insects were fed after coating 50 µg cholesterol on small pieces of green grass. In controls, no cholesterol was given to insects. After 6 h, insects were dissected to collect midgut, ovary, and testis. Small segments of tissues (1 mm^3) were fixed for 24 h at room temperature in PBS containing 2% w/v paraformaldehyde and 0.2% glutaraldehyde. The isolated tissues were cut into small pieces and preserved in formalin overnight. Fixed tissues were washed in de-ionized water and subjected to dehydration in different grades of alcohols and finally to xylene. The tissue pieces were embedded in molten wax. After embedding, tissue blocks were made and subjected to microtome sectioning.

Positive control 1

In positive control, paraffin sections (3 to 4 µm) were cut using a microtome (Weswox Optik). These sections were stretched on microscopic glass slides, de-parafinized and hydrated (100, 90, 70, 50%, water). Sections were put in hematoxylin, solution (0.3%), washed in water and dehydrated up to 90% alcohol and put in eosin for better differentiation. Sections were passed through 90, 100 and 100% alcohol + xylene and cleared in xylene and mounted in D.P.X.

Positive control 2

Another positive control was set in which semi-thin sections were stained with Sudan black-B by using the method of Bayliss and Adams (1972).

Negative control

Semi thin sections were de--parafinized and passed through different grades of alcohols as used in previous controls. No treatment was given to these sections. These were simply dehydrated and mounted in DPX

Test 1: primary and secondary antibody treatment

Semi-thin sections were incubated in PBS (10 mM sodium phosphate, 150 mM NaCl pH 7.4) with 0.1% BSA and 0.1 gelatin in cold water for 3 h. Sections were permeabilized with 0.2 of Triton X-100 in PBS for 30 min. Then these sections were rinsed in PBS and treated with 70, 90, 100, 90% ethanol for 2 min. These sections were incubated with 2.25% gelatin in 0.1 M PBS for 1 h. These were further incubated in primary antibody over night at 4°C. The next day, tissues were rinsed in PBS and fixed for 5 min in bromine water. Sections were rinsed in PBS and incubated for 4 h in HRP labeled secondary antibody (1:200 dilution). Sections were washed in PBS. After proper dehydration slides were cleared in xylene and mount in D.P.X.

Test 2: treatment with primary antibody

Semi thin sections were incubated in PBS (10 mM sodium phosphate, 150 mM NaCl pH 7.4) with 0.1% BSA and 0.1% gelatin in cold water for 3 h. Small drops (35 µl sized) of PBS+ 4% BSA were put on each section and incubated at 4 ^{0}C for 30 minutes. Sections were washed twice with PBS and treated with 25 µL of purified primary antibody (1:500 dilutions in PBS) and incubated overnight at 4°C. Next day tissue sections were washed properly with PBS. It was then allowed to incubate for 4 h after treating them with few drops of (25 µL) diluted (1:200) HRP-labeled secondary antibody. It was washed in PBS and fixed in 4% glutaraldehyde for 5 min in 0.1 M Na_2PO_4 phosphate buffer. Sections were rinsed in water, dehydrated in graded alcohol properly and mounted in DPX.

Treatment with fetal bovine serum (normal serum)

Semi-thin sections were incubated in PBS (10 mM sodium phosphate, 150 mM NaCl pH 7.4) with 0.1% BSA and 0.1% gelatin in cold water for 3 h. Small drops (35 µL sized) of PBS+4% BSA were put on each section and incubated at 4°C for 30 min. Sections were washed twice with PBS. After washing, 25 µL sized drops of normal serum (Fetal Bovine Serum) were put on the sections and allowed to incubate overnight at 4°C. The next day sections were washed properly with PBS. Sections were fixed in 4% glutaraldehyde for 5 min in 0.1 M phosphate $NaPO_4$ and rinsed in water, dehydrated and mount.

Immuno-florescent labeling

Tissue sections were saturated in PBS containing 1.5% BSA for 10 min at room temperature. Sections were washed for 10 min in fresh PBS. Tissue sections were incubated with pAb anti-CBP for 1 h at room temperature in 1:100 dilutions. These sections were washed in PBS and incubated with the TRITC labeled anti-rabbit immunoglobulin diluted 200 times in PBS containing 0.5% BSA for 30 min at room temperature. Tissue sections were further rinsed three times in PBS buffer and then mounted in DPX and photographed under 2-M florescent microscopes. Control experiments were carried out using pAbs anti-CBP as primary antibodies.

RESULTS

Purification and characterization of cholesterol binding proteins

Elution pattern of cholesterol binding proteins on gel filtration column gave two peaks at 260 and 280 nm, first soon after the void volume from fractions 31 to 50 and the second in fractions 61 to 71 (Figure 1a and b). Further, both peaks were compared with proteins obtained in each fraction. Interestingly, both peaks obtained coincided with the protein peak obtained at 640 nm absorbance. As a result the first peak contained large amount of cholesterol with less amount of protein but the second peak associated large amount of cholesterol with lesser amount of protein that represents specific binding (Figures 1c to e).

Molecular weight determination

Molecular weight of CBPs was determined by gel filtration chromatography. The fractionation of midgut proteins presented associated large amount of cholesterol. These proteins showed molecular weight in a range of 34 to 70 kDa (Figure 1f). Further, proteins eluted in fractions 61 to 71 were pooled and their molecular weights were determined by electrophoresis.

Production of polyclonal antibodies against cholesterol binding proteins

After purification, antibody recovery was obtained by 1.10 mg/ml in crude antiserum. It was partially purified by octanoic acid and consequently ammonium persulphate precipitation. % yield obtained in the precipitation was 70.90%. After diffusion of CBPs (antigen) and pAb anti-CBP in agar gel, and due to its close interaction, an immune complex precipitated in the gel and gave a thin visible white sickle shaped line of precipitation due to equivalence of both substances or formation of a concentration gradient (Figures 2a to d). It shows a visual signature of antigen recognition both in Ouchterlony test and precipitation ring test (Figures 2a to d).

Electrophoretic separation of cholesterol binding proteins

When midgut and pooled fractions were subjected to 4 to 25% gel electrophoresis, it gave molecular weight of CBPs in a range of 68 kDa (Figure 3). Further, localization of proteins electrophoresis gels were stained with Sudan black-B. It showed specific binding to the CBPs and stained only cholesterol associating proteins.

SDS-PAGE immuno blotting

Immunoblotting technique confirmed CBP expression in midgut tissue of locusts. Immuno-blotting revealed similar band at about 68 kDa in both the SDS gel and immunoblotting in insect midgut. In cholesterol unfed insects, these bands were totally absent, which was confirmed by the primary antibody interaction. The immuno-reactivity was found in the same band when allowed to incubate with primary and HRP labeled secondary antibody (Figure 4).

Immunocytochemical localization of CBPs

Semi thin sections of insect midgut, ovary and testis were fixed in picric acid, 0.2% formaldehyde for 24 h. These tissue sections were allowed to be treated with primary and secondary antibody labeled with HRP. Semi-thin sections of midgut tissues of *S. gregaria* were incubated with pAbs anti-cholesterol binding proteins (CBPs) and in HRP conjugated goat anti-rabbit immunoglobulin. Following test and control experiments were conducted and results were obtained in tests and controls.

Positive control 1

In positive control, semi thin sections of midgut, ovary and testis were processed using the same method and stained with Hematoxylin. For better differentiation, tissue sections were also stained with eosin and passed through various grades of alcohol starting from 90, 100 and 100% alcohol and xylene. These sections revealed the general morphology of the cells and localized presence of few stained granules (Figures 5e, 6a and 7e).

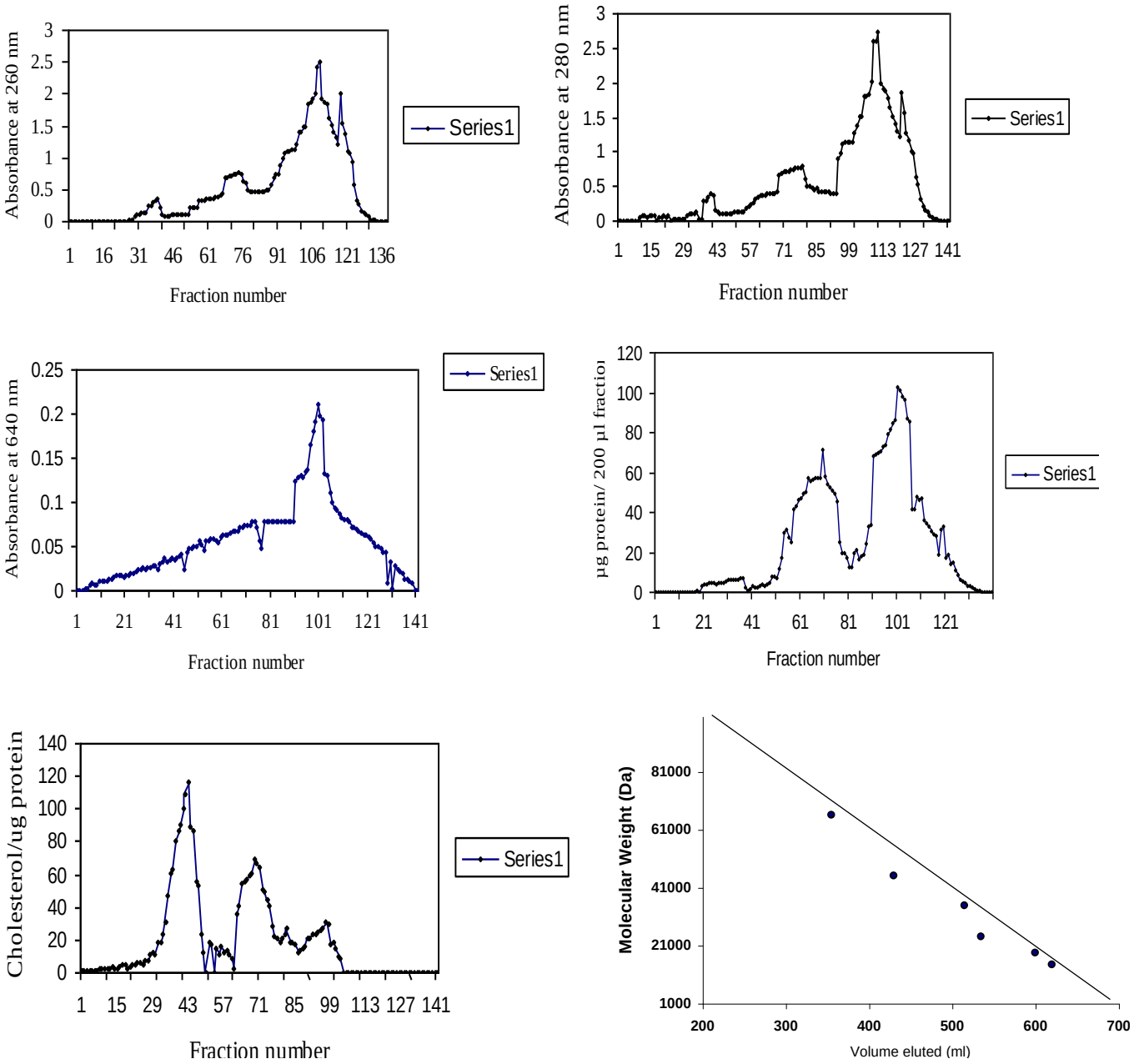

Figure 1. Elution pattern of PBS extractable proteins of *S. gregaria* midgut chromatographed on Sepharose CL-6B column. (a) absorbance at 260 nm; (b) absorbance at 280 nm; (c) absorbance at 640; (d) µg protein/ 200 µl fraction; (e) specific activity ; (f) Standard proteins chromatographed on Sepharose CL-6B 200 column for determining the molecular weights of cholesterol binding proteins isolated from *S. gregaria.* Proteins used were bovine albumin mol. wt 66,000, egg albumin mol. wt. 45,000, pepsin mol. wt. 34,700, trypsinogen mol. wt. 24,000, beta lactoglobulin mol. wt 18,400 and lysozyme mol. wt. 14, 300. Elution volumes of unknown proteins were compared with log values on the X-axis for estimation of molecular weights.

Positive control 2

In positive control semi-thin midgut sections were de-parafinized and dehydrated up to 100% alcohol and stained with Sudan black-B dye in which midgut epithelium was found to be more visible which was composed of columnar microvillus cells. These showed cholesterol localized regions in form of droplets. Sudan black-B specially stained the cholesterol droplets in form of black granules, which gave positive binding of cholesterol at appropriate binding sites (Figure 8).

Negative control

In the negative control, no reaction was done to localize the CBPs in midgut, ovary and testis tissue sections. It gave no reaction or stains because no treatment of any

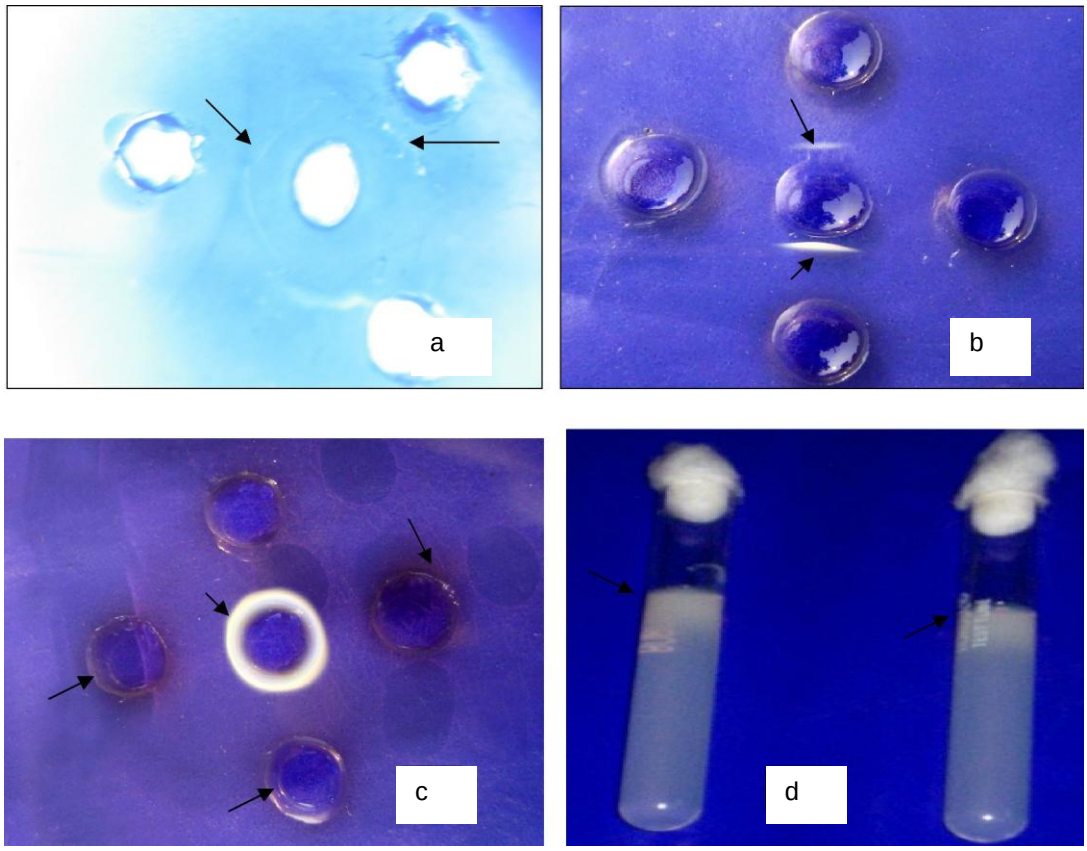

Figure 2. Confirmation of polyclonal antibodies generated against cholesterol binding proteins (CBPs) of locust. a and b showing results of immunodouble diffusion test; c and d precipition ring test; d agglutination test respectively.

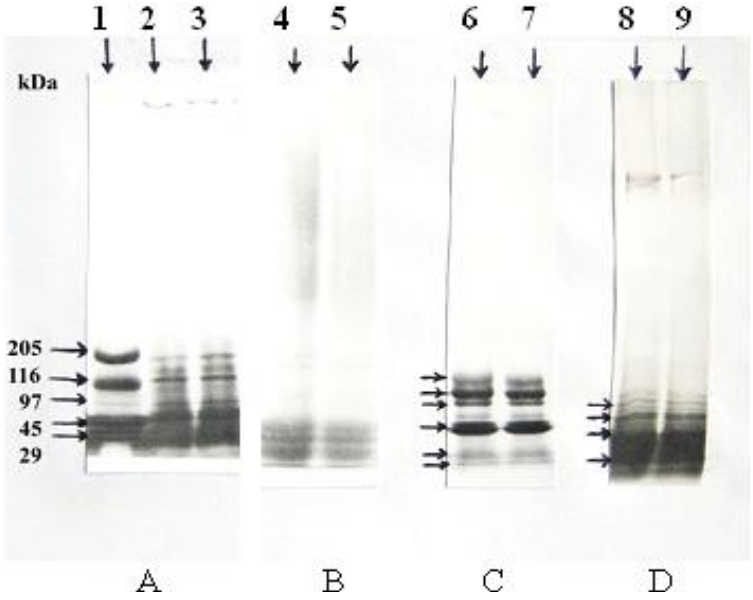

Figure 3. Electrophoretic separation of midgut proteins of male and female *S. gregria* using 4-25% native gel electrophoresis (A). Lane 1 molecular weight marker; lanes 2 and 3 midgut protein from supernatant. **(B)** Lane 4 and 5 fat body proteins; **(C)** Lanes 6 and 7 ovary proteins. **(D)** Lane 8, 9 testis proteins. 70 µl of sample was subjected to electropho-resis and gels were stained with Commassie brilliant blue R-250.

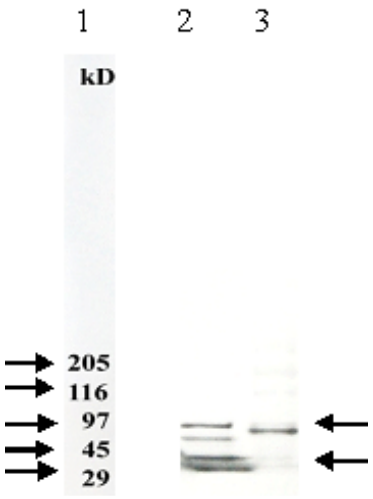

Figure 4. Immunoblotting of CBP. Supernatant of midgut homogenate was subjected to SDS-PAGE (12%) analysis followed by immunoblotting. Lane 1 showing molecular mass markers (B-Genei); lane 2 pAbs and anti-CBP were found to react specifically with a 68 kDa protein band corresponding to that of that CBP.

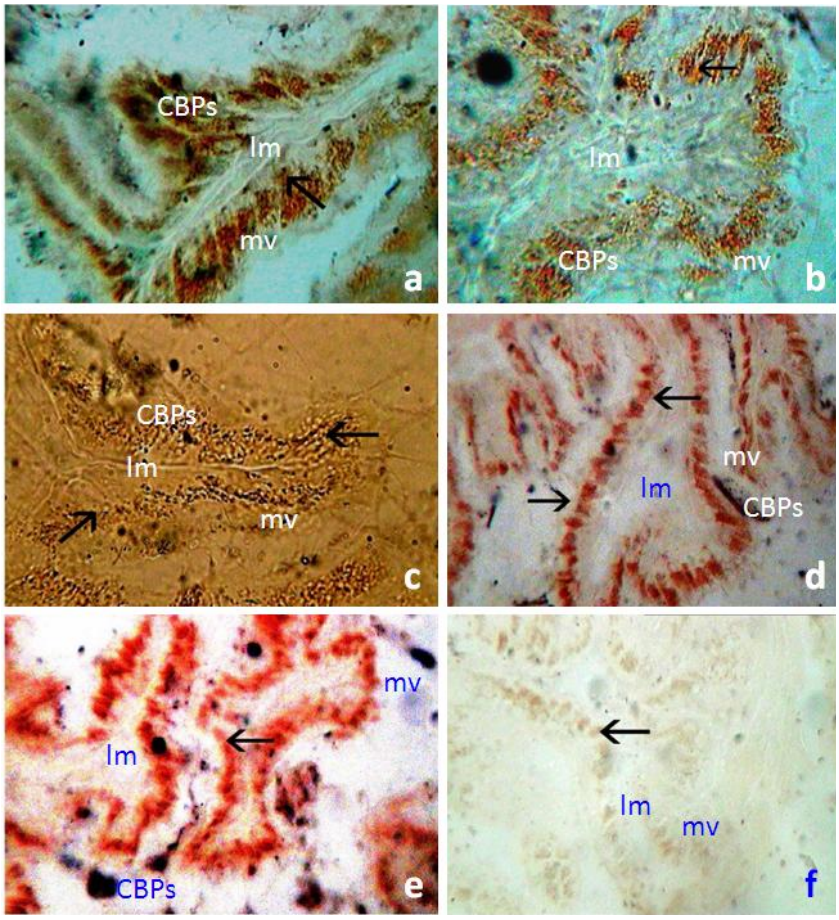

Figure 5. Semi-thin sections of midgut epithelium of *S. gregaria* showing immunocytochemical localization of cholesterol binding proteins after ingestion of cholesterol. Immuno-reactivity is localized in microvillus cells in control and tests. (a)Treatments expressed the binding of primary and secondary antibody (1:200) with HRP labeling to cholesterol binding site. Proteins holding cholesterol gave color reaction; (b) Similar treatment showing higher binding; (c) treatments expressed only primary antibody binding; (d) positive control-tissue sections treated with normal serum; (e) Control sections stained with hematoxylin and eosin for general morphology and (f) Negative control-tissue sections without any staining and any treatment.

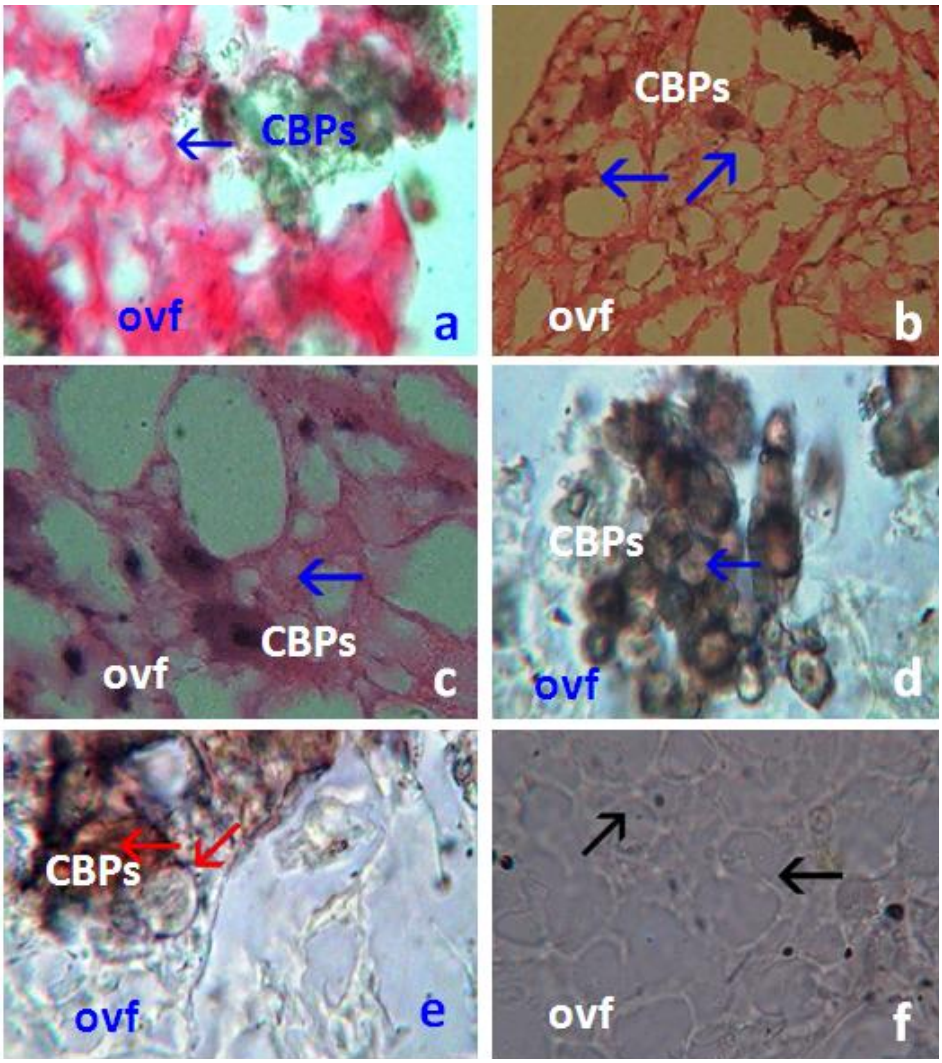

Figure 6. Semi-thin sections of ovarian tissue of *S. gregaria* showing immunocytochemical localization of cholesterol binding proteins after ingestion of cholesterol. Immuno-reactivity is localized in ovarian follicle cells in control and tests. (a) Stained with hematoxylin and eosin; (b) stained with hematoxylin for general physiology; (c) stained with eosin for general morphology higher magnification; (d) primary and secondary antibody HRP labeled; (e) positive control-tissue sections treated with normal serum; (f) negative control-tissue sections without any staining and any treatment.

substrate or staining material was given. It was proceeded only to pre fix the structural microscopic observations of midgut epithelium for better comparison or results (Figures 5f, 6f and 7f).

Test 1

Tissue sections when treated with purified primary antibody (1:500 dilutions in PBS) and HRP labeled secondary antibody (1:200 dilutions in PBS) reaction and cholesterol binding sites were visualized clearly in form of granules. These were found deposited in apical areas of epithelial cells (mucosal cells) and mostly near the site of absorption of cholesterol (Figure 5a and b).

Test 2

Tissue sections when incubated in PBS with 0.1% BSA and 0.1% gelatin in cold water for 3 h. Sections were permeabilized with 0.2% Triton-X 100 in PBS for 30 min. These sections were washed in PBS and passed through various grades of alcohols. Sections were treated with primary antibody, which showed positive binding to cholesterol (Figure 5c).

Tests 3

Tissue sections were treated with normal serum. Serum components were found associating with cholesterol

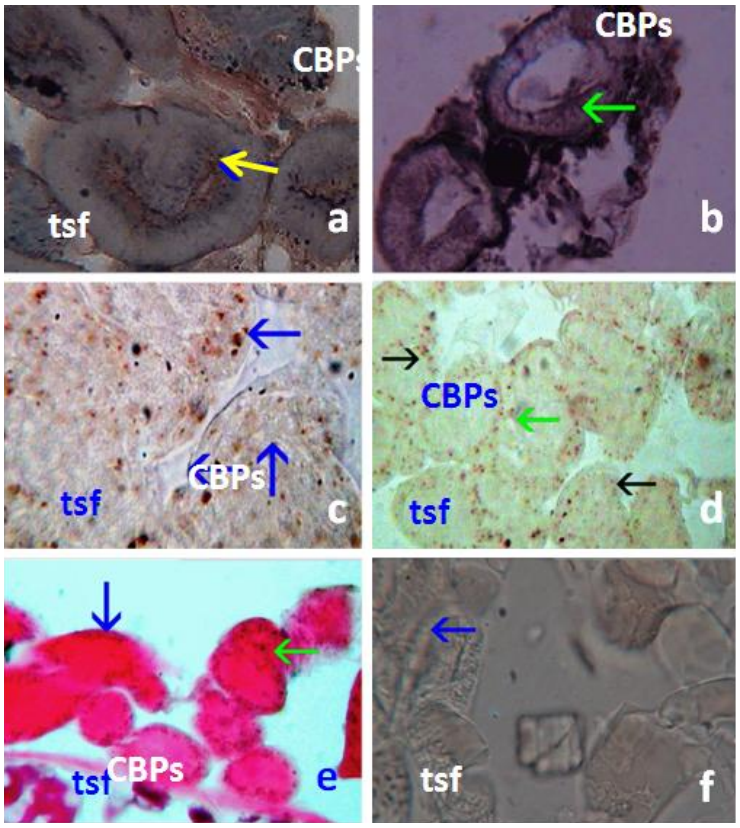

Figure 7. Semi-thin sections of testicular tissue of *S. gregaria* showing immunocytochemical localization of cholesterol binding proteins after ingestion of cholesterol. Immuno-reactivity is localized in testicular follicle cells and vas deferens. (a) Tissue sections stained with Sudan black B; (b) Testicular follicle and vas deferens stained with Sudan black B; (c) tissue sections treated with primary antibody and secondary antibody labeled with HRP; (d) positive control- tissue sections treated with normal serum; (e) tissue sections stained with hematoxylin and eosin; (f) negative control-tissue sections without any staining and any treatment.

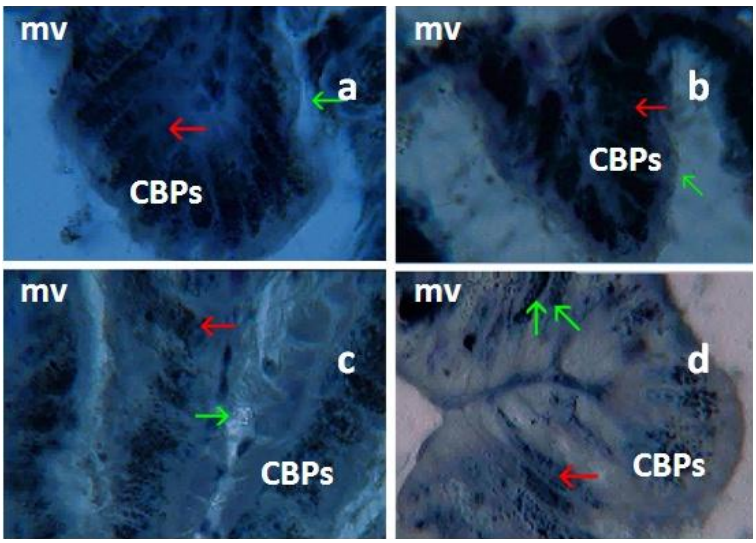

Figure 8. Semi-thin sections of midgut epithelium of *S. gregaria* showing localization of cholesterol binding proteins in microvillus cells. Tissue sections were stained with Sudan Black B method of Bayliss and Adams (1972).

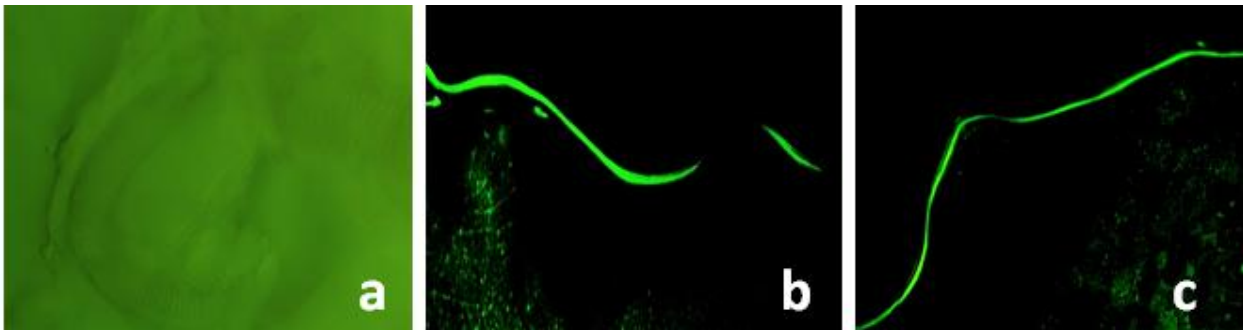

Figure 9. Semi-thin cross sections of the midgut wall showing the basal region of the epithelium. Insects were provided cold cholesterol with diet. 8 h later, their midgut portions were isolated, thoroughly washed with insect culture medium and then incubated with primary antibody and then TRITC labeled secondary antibodies. (a) 15 min; (b) 60 min; (c) In control no treatment was given to the tissues.

binding proteins and showed positive binding in form of granules in the apical areas of micro-villous cells (Figure 5d).

Immuno-fluorescent labeling

Immunocytochemical fluorescent labeling of midgut tissue sections exhibited strong fluorescence in side apical region on microvillus cells midgut epithelium. The fluorescence was found restricted to the pattern of an irregular lines partly connected to a network.

Semi-thin cross sections of midgut wall was stained with anti-CBPs and Sudan black–B resulted in an irregular staining of the midgut epithelial cells where CPBs were confirmed in the apical region of microvillus cells and in the lumen. However, the basal lamina exhibited stronger fluorescence in side of the midgut epithelium. The fluorescence was found restricted to the pattern of irregular lines partly connected to a network (Figure 9) which coincided with the out lines on the midgut epithelial cells and proved that the CBPs are localized along the baso-lateral region of microvillus cells.

The primary antibody generated for the immunocytochemical localization has shown specific binding to CBPs when it was revealed by Ouchterloy test and western blot technique. The western blotting had also revealed the presence of CBPs in the midgut epithelium showing specific binding with cholesterol.

DISCUSSION

The results from the present investigation clearly demonstrate that cholesterol binding proteins are responsible for cholesterol absorption in insect midgut. These are histochemically localized in midgut microvillus cells of *S. gregaria* (Forskal) after treatment. CBPs were isolated from midgut tissue of locusts and purified on a gel filtration column. Chromatogram obtained from gel filtration chromatography of locust midgut proteins resolved two major peaks, which associated a large amount of cholesterol. First protein peak was obtained soon after the void volume in fraction number 31 to 50, while second peak was found between fractions 61 to 71 (Figure 1a and b). When eluted fractions were analyzed on UV-spectrophotometer at 260 and 280 nm absorbance; it also gave two peaks soon after the void volume. First peak was showing maximum association of cholesterol with protein in fractions 20 to 50, while second was associating a large amount of cholesterol between fractions 61 to 71 (Figures 1c to e).

Molecular weights of these proteins were determined after elution of standard proteins of known molecular weight at same flow rate. These proteins were showing molecular weight between 34 to 70 kDa (Figure 2f). It was probably HDLp, which carries the cholesterol to various organs (Ioannoue, 2007). When pooled fractions 64 to 71 were applied on SDS gel electrophoresis, a protein band was obtained associating a large amount of cholesterol (Figure 3). It was further confirmed in immune-blot transfer analysis in which a CBPs and pAb anti-CBP interaction gave a positive while rests of the bands in the same lane remain undetected (Figure 4). Further, it was histochemically localized in treatments (tests) in apical zone of midgut epithelial cells which displayed positive labeling of CBPs in form of spherical protein bound cholesterol granules (photograph). More specifically, in tests where tissue sections were treated with primary antibody (pAbs anti-CBP) and HRP labeled secondary antibody, they have displayed specific binding to CBPs in apical areas, which appeared in zymogene-like granules. These granules could be cholesterol protein complexes, which were formed after immuno-reactivity (Photograph). It confirms presence of cholesterol binding proteins in midgut epithelial cells. However, cholesterol depositions were localized in form of large dense granules having irregular shape and size.

In another test, in which tissue sections were treated with normal serum also displayed few granules at the same site but lesser binding in comparison to HRP labeled antibodies. In another test, midgut tissue sections were incubated with primary antibody alone. For compa-

rative binding, tissue sections were stained with Sudan black-B, which also displayed dark color granules of cholesterol and CBPs complexes and demonstrate cellular localization of cholesterol (Figure 8). In all the cases, association of these proteins was found associated to some ligand at the place, where cholesterol binding protein and cholesterol complex granules were found. Further, cellular localization of CBPs and their homogenous distribution in the apical portion of microvillous cells of midgut confirms the role of these proteins in cholesterol absorption. This study also inferred that midgut epithelial cells are major sites of cholesterol absorption because most of the binding sites were found in apical region of these cells. Further, these structural locations of granules in midgut epithelial cells of *S. gregaria* were examined under light microscope and photographed. In controls, cholesterol bound CBPs granules were totally absent because no antibody was used to confirm the specific binding. Furthermore, cholesterol immunohistochemical localization of CBPs was also done in ovary and testis, which also gave positive results after antigen and antibody interaction. It also confirms presence of CBPs in other tissues, and its important role in cholesterol transport in insects. (Figures 6 and 7). Similarly, few fatty acid and lipid carrier proteins were immunocytochemically localized in intestine, hemolymph and fat body of *Heliothis zea* by Kuthiala and Ritter, (1988), flight muscles of locusts (Li and Powanall, 2001; Xinemi and Haunerland,1994) and midgut tissue of *Manduca sexta* (Stahl et al.,1999), Hamster cells (Bertolotti and Spady, 2001) and human intestine (Mayer et al., 1985).

HRP labeled rabbit anti-goat antibody specifically stained CBPs showed stronger reactivity in midgut epithetlial cells in Western blot (Figure 4). Further, localization of CBPs was confirmed by using fluorescent labeling in epithelial cells, which also prove CBP might be an intracellular protein and involve in cholesterol absorption. Before absorption, cholesterol may bind to these proteins (Upadhyay and Agarwal, 2007) and release in to the hemolymph from where it transported to different organs of insects (Sakai and Rawson, 2001). These sterol carrier proteins are also characterized in different insects like cotton bollworm, *Helicoverpa armigera* (Du et al., 2012), caterpillar of *Helicoverpa zea.* (Jing et al., 2012), *Spodoptera litura* (Guo et al., 2009), and *Manduca sexta* (Jouni et al, 2002) and immunocytochemically localized in insect tissues (Dyer et al., 2003; Horton and Shimomura, 1999). It suggests that CBPs are very similar to SCPs and FABPs in function and might have belong to family of sterol carrier or lipid carrier proteins which are evolved in different insect groups during long evolution. These proteins also show structural diversity in higher invertebrates and vertebrates and perform lipid transport functions (Clarcke et al., 2000; Huang et al., 2002) and act as sterol regulatory element binding protein 1 in the rodent and primate brain (Ong et al., 2000) and perform cytoskelatal function in nerve cells (Caceres et at, 1986). These proteins essentially take part in cholesterol absorption, trafficking and tissue distribution inside insect body (Zouari et al., 2006, Upadhyay and Agarwal, 2007). More exceptionally, few other proteins like lipase (Roberts et al., 1986; Dupont et al., 1992), and 3-beta-hydroxysteroid dehydrogenase (Wouters et al., 1995) were also immunocytochemically localized in animal tissues. These proteins also perform similar lipid transport functions. Therefore, it is important to note that transport functions of CBPs in present study are quite similar to sterol carrier proteins characterized in higher vertebrates previously. Hence, further progress is needed to explore major role of these proteins in different cell types for intracellular trafficking of cholesterol and other lipids as well as its absorption and transport in insects.

Conclusion

On the basis of experiments conducted and results obtained, it can be concluded that CBPs are main carrier vehicles of cholesterol, which facilitate absorption in midgut epithelial cells. It also confirms that before absorption, cholesterol complexed with CBPs and internalized inside epithelial cells.

Conflict of Interests

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

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