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Review

Trichoderma as biological control weapon against soil borne plant pathogens

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The genus of *Trichoderma* is widely applied for the biocontrol of phytopathogenic fungi in agriculture sector. Moreover, *Trichoderma* species are also excessively exploited in different industrial purposes due to their production of important lytic enzymes such as chitinases, glucanases and proteases. Several genetic improvement trials are carried out for maximizing the role of *Trichoderma* as biological control agents via mutation, protoplast fusion, and genetic transformation. This review highlights the mode of action of *Trichoderma* against pathogenic fungi, potential applications in different fields of life, and the recent genetic improvement trials for increasing the antagonistic abilities of this fungus as biological control agent.

Key words: *Trichoderma*, antagonism, lytic enzymes, genetic improvement.

INTRODUCTION

Farmers around the world need the chemical pesticides to control the plant disease pathogens in order to maintain the quality and redundancy of agricultural products (Junaid et al., 2013). It was estimated that 37% of crop loss is due to pests, of which 12% is due to pathogens (Sharma et al., 2012). On the contrast, the excessive and the misuse of pesticides over the past decades caused environmental pollution and several health problems in addition to their expensive costs for developing countries. Moreover, the long term use of chemical pesticides can lead to development of certain resistant organisms (Naher et al., 2014). Recently, the world attention resort to find sustainable, safe and ecofriendly alternatives. Biological control agents (BCA) refer to the utilization of some living microorganisms to suppress the growth of plant pathogens (Pal and Gardener, 2006). In other words, biological control means the use of beneficial organisms, their genes, and/or products to reduce or suppress the negative effects of plant pathogens (Junaid et al., 2013). Currently, several biocontrol agents have been recognized and are available as bacterial agents for example *Pseudomonas, Bacillus*, and *Agrobacterinum* and as fungal agents such as *Trichoderma, Aspergillus*, *Gliocladium*, *Ampelomyces*, *Candida*, and *Coniothyrium* (Naher et al., 2014). *Trichoderma* is one of the famous filamentous fungi widely distributed in the soil, plant material, decaying vegetation, and wood (Gajera et al., 2013). The

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Trichoderma genus are related to the order of Hypocreales, family of Hypocreaceae and the genus have more than 100 phylogenetically defined species (Kumar, 2013). Among the common species of *Trichoderma* are *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma pseudokoningii*, *Trichoderma viren*, and *Trichoderma viride*.

Trichoderma is considered an excellent biocontrol agent model due its high ability to multiply, spread, easy to isolate and culture (Pandya et al., 2011). *Trichoderma* strains can act as biocontrol agents against fungal phytopathogens such as *Phythium*, *Phytophthora*, *Botrytis*, *Rhizoctonia,* and *Fusarium* through several mechanisms including competition for nutrients and space, antibiosis and induction of plant defensive mechanisms and mycoparasitism (Benítez et al., 2004). It was shown that *Trichoderma* can use one or more of these mechanisms according to type of fungus and the environmental conditions such as temperature, pH, and nutrient concentrations (Gajera et al., 2013). Recently, the number of products based on *Trichoderma* found in the international market is increasing with more than 250 products (Woo et al., 2014). This review highlights the different mechanisms of *Trichoderma* as biological control agents and the genetic tools to improve these activities.

HISTORY OF *TRICHODERMA*

The first description of *Trichoderma* as a fungus was by Persoon in 1794 who described this fungus as appearing like mealy powder enclosed by a hairy covering. It was reported that the genus of *Trichoderma* contain only one species, namely *T. viride* (Bisby, 1939). Then Rifai (1969) distinguished nine species using the analysis of morphological characteristics: *T. harzianum* Rifai*, T. viride, Trichoderma hamatum* (Bonord.) Bainier*, T. koningii* (Oudem.) Duché *&* R. Heim*, Trichoderma polysporum* (Link) Rifai*, Trichoderma piluliferum* J. Webster *&* Rifai*, Trichoderma aureoviride* Rifai*, T. longibrachiatum* Rifai, and *Trichoderma pseudokoningii* Rifai (Błaszczyk et al., 2014). Recently, 104 species of *Trichoderma* have been registered internationally (Pandya, 2011). It must be mentioned that Weindling (1932) referred for the first time the importance of *Trichoderma* as bioagents.

TRICHODERMA BIOCONTROL MECHANISMS

Competition

Since *Trichoderma* strains grow rapidly in the soil due to their natural resistance to many toxic compounds, including herbicides, fungicides, and pesticides; this gave it a superior ability to colonize, take up soil nutrients, and therefore starvation of other organisms from nutrition (Chet et al., 1997; Benítez et al., 2004). Competition for space and for nutrients such as carbon and nitrogen is an important feature of *Trichoderma* antagonism (Vinale et al., 2008). Sivan and Chet (1989) demonstrated that competition for nutrients is the major mechanism used by *T. harzianum* to control *Fusarium oxysporum f. sp. melonis*. It is well known that iron uptake is essential factor for viability of pathogen in soil, so that some *Trichoderma* strains produce highly efficient siderophores to chelate iron and stop the growth of other fungi (Chet and Inbar, 1994). Furthermore, the ability of *Trichoderma* to obtain ATP from the metabolism of several substrates such as cellulose and glucan give it a competitive advantage than the other pathogens. Ferre and Santamarina (2010) showed that colonies of *T. harzianum* inhibited the growth of *Fusarium culmorum* strains in different environmental conditions and the macroscopic analysis of the petri plates revealed that *T. harzianum* competed *F. culmorum* for space and nutrients.

Mycoparasitism

Mycoparasitism is a very complex process in which *Trichoderma* recognizes signals from the host fungus, coils around host hyphae and host penetration. The lytic enzymes of *Trichoderma* such as chitinases, glucanases and proteases degrade the host cell wall and kill them (Sharma et al., 2012). It well known that the cell wall of *Pythium* species is composed of cellulose (Figure 1), while chitin is the main structural component of *Rhizoctonia solani* cell walls (Bartinicki-Garcia, 1968; Farkas, 1990; Sivan and Chet, 1989). Moreover, *Trichoderma* are good producer of chitinases that hydrolyze the glycosidic bonds between the N-acetyl glucosamine residues of chitin (Agrawal and Kotasthane, 2012); also, cellulases which hydrolyse β-1,4 glucans (Nevalainen and Penttilä, 1995) and these enzymes are among the most effective weapons for plant diseases biological control. It was shown that *Trichoderma* species are the most common mycoparasitic and saprophytic fungi that have high ability for colonization and attack a great variety of phytopathogenic fungi responsible for important diseases of major economic crops worldwide (El-Hassan et al., 2012*)*.

Howell (2003) obtained transformants of *T. harzianum* T3 that produce a variety of cellulases, which make this isolate very effective in the control of *Pythium ultimum* on cucumber seedling than the wild type. Furthermore, Limon et al. (1999) obtained transformants of *T. harzianum* strain CECT 2413 that overexpressed chitinase (*chit33*) and these transformants were more effective in inhibiting the growth of *R. solani* as compared to the wild type.

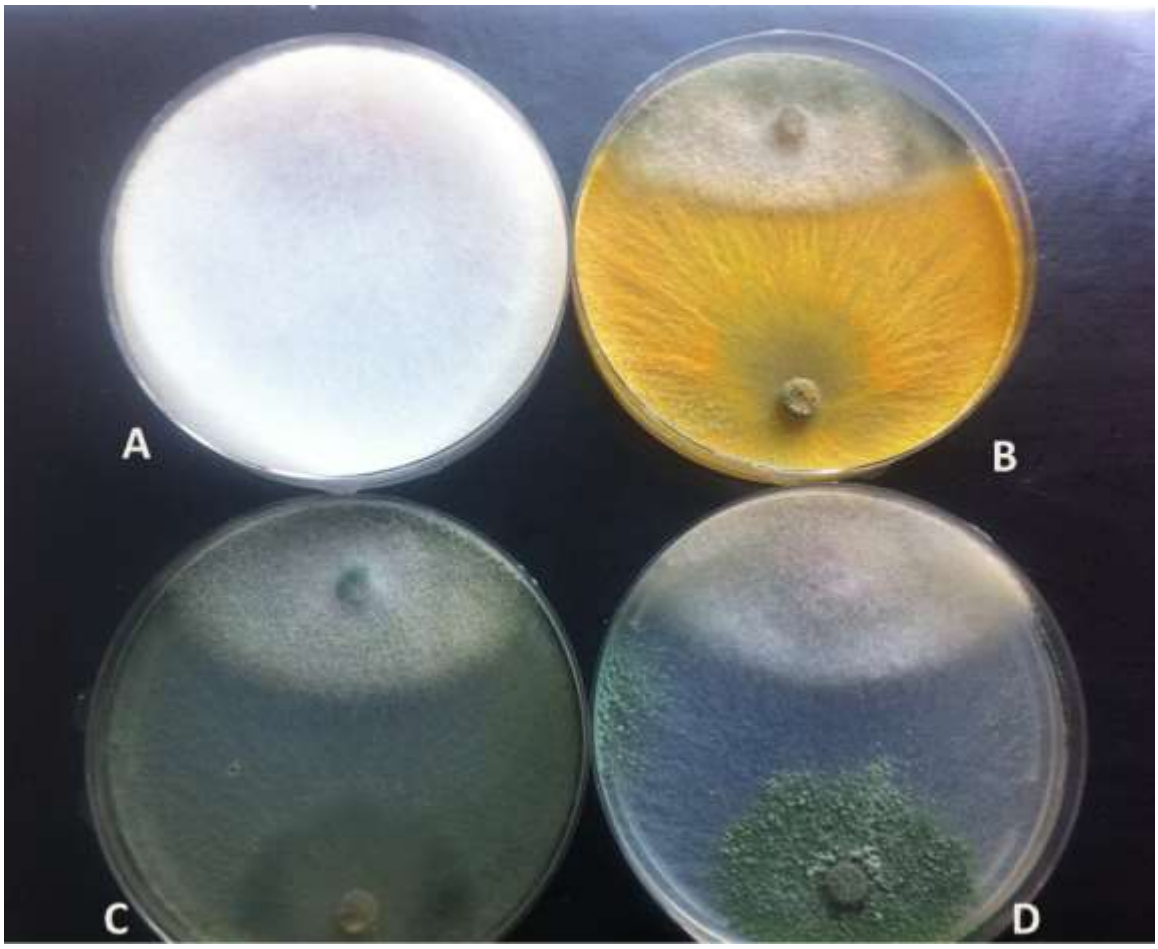

Figure 1. The antagonism of *Trichoderma* spp. against *Pythium* spp. (A) The fungus of *Pythium* spp. while B, C and D different species of *Trichoderma* overgrowth *Pythium* spp.

Antibiosis

Trichoderma can produce low molecular weight diffusible compounds or antibiotics that inhibit the growth of other microorganisms. There are several metabolites or antibiotics secreted from *Trichoderma* against their pathogens such as: harzianic acid, tricholin, peptaibols, 6-penthyl-α-pyrone, viridin, gliovirin, glisoprenins, and heptelidic acid (Gajera et al., 2013). Peptaibols are a large family of antibiotic peptides and *Trichoderma* can synthesize more than 190 of these compounds. Trichokonin VI (TK VI) of *T. pseudokoningii* is one of the peptaibols that can induce an extensive apoptotic programmed cell death in plant fungal pathogens such as *F. oxysporum* (Shi et al., 2012).

Sadykova et al. (2015) tested the antibiotic activity in 42 strains of 8 species of the *Trichoderma* genus (*Trichoderma asperellum, T. viride, T. hamatum, T. koningii, T. atroviride, T. harzianum, T. Citrinoviride*, and *T. longibrachiatum*) isolated from Siberian. It was shown that these species differ in the degree of their antibacterial and antifungal activity and the strain *T. citrinoviride* TV41, exhibited high activity and a wide range of actions against the pathogenic fungi of the *Aspergillus* and *Candida albicans* genus and bacteria, including methicillin resistant *Staphylococcus aureus*. The authors expected that peptaibols are probably the most active compounds in the strain culture extracts according to mass and IR spectrometry data. Vinale et al. (2014) showed that the pyrone 6-pentyl-2H-pyran-2-one is a metabolite purified from the culture filtrate of different *Trichoderma* spp. (*T. viride, T. atroviride, T. harzianum* and *T. koningii*) and has shown both *in vivo* and *in vitro* antifungal activities towards several plant pathogenic fungi. In addition, Ghisalberti et al. (1990) demonstrated that the biocontrol efficacy of *T. harzianum* isolates against *Gaeumannomyces graminis var. tritici* is related to the production of pyrone-like antibiotics. Furthermore, Howell (1999) reported that strains of *Trichoderma virens* (P group) produce the antibiotic gliovirin which is very active against *P. ultimum,* while the Q group of these strains can produce gliotoxin, which is very active against

R. solani.

Induction of plant growth and defense

Trichoderma spp. are well-known for their ability to promote plant growth and defense. *Trichoderma* can increase root development, shoot length, leaf area and therefore crop yield via colonization of plant roots, proliferation of secondary roots and solubilizing several nutrients as P and Fe to plants (Hermosa et al., 2012). The previous studies showed that *Trichoderma* can produce gluconic and citric acids that decrease the soil pH, enhance the solubilization of phosphates, micronutrients, and mineral components such as iron, magnesium, and manganese (Benitez et al., 2004; Harman et al., 2004b; Vinale et al., 2008). It was noted that the bean plants treated with *T. harzianum* T019 always had an increased size respect to control. In addition, this strain induced the expression of plant defense-related genes and produced a higher level of ergosterol, indicating its positive effects on plant growth and defense in the presence of the pathogen (Mayo et al., 2015). Moreover, the roots of maize plants treated with *T. harzianum* strain T-22 were about twice as long compared to untreated plants after several months from treatment (Harman, 2004a).

Saravanakumar et al. (2016) showed that *Trichoderma* cellulase complexes trigger the induced systematic resistance (ISR) against *Curvularia* leaf spot in maize by increasing the expression of genes related to the jasmonate/ethylene signaling pathways. Furthermore, Rao et al. (2015) suggested that treatment of legume seeds (*Cajanus cajan*, *Vigna radiate* and *Vigna mungo*) with *T. viride* induces systemic resistance by reprogramming defense mechanisms in these legumes. Reprogramming alleviated the levels of defense enzymes (PO, PPO and PAL), ROS ($O_2^{\bullet-}$, H_2O_2, OH•), antioxidant enzymes (CAT, SOD), scavenging activity of antioxidant enzymes in response to oxidative stress induced by *F. oxysporum* and *Alternaria alternata*. This mechanism helps in developing resistance in plants and therefore protect from pathogens. *Trichoderma* metabolites may also increase disease resistance by triggering systemic plant defence activity and/or enhance root and shoot growth (Vinale et al., 2014).

OTHER APPLICATIONS OF TRICHODERMA

In addition to their important roles as biocontrol agents, plant growth promoter and defense, there are some other applications for *Trichoderma* in different fields as shown in Figure 2.

Bioremediation of contaminated soils

Trichoderma strains play an important role in the bioremediation of soil contaminated with pesticides and herbicides as consequence of their high abilities to degrade a wide range of insecticides: organochlorines, organophosphates, and carbonates (Kumar, 2013). Moreover, since *Trichoderma* is a potent producer of hydrolytic and industrially important enzymes, like cellulases and chitinases, this make *Trichoderma* spp. highly resistant to a wide range of toxicants, heavy metals, tannery effluents, and harmful chemicals like cyanide (Hasan, 2016). The above advantages make them an ideal fungal genus in bioremediation of toxic pollutants. Previous studies showed that *Trichoderma* spp. can remove and accumulate the various heavy metals such as copper, zinc, cadmium, and arsenic through sorption and biovolatilization (Yazdani et al., 2009; Srivastava et al., 2011; Zeng et al., 2010). Teng et al. (2015) showed that *T. reesei* FS10-C enhances the phytoremediation ability of Cd-contaminated soil by the hyper accumulator *Sedum plumbizincicola* and also increases soil fertility. Moreover, it was reported that each *T. virens PDR-28* and *T. pseudokoningii* increased the dry biomass and Cd accumulation of maize and pearl millet, respectively as compared to the control (Babu et al., 2014; Bareen et al., 2012). Furthermore, Arfarita et al. (2013) reported that the isolate of *T. viride strain FRP3* was able to grow in culture medium containing the herbicide glyphosate as the sole phosphorus source. This was coupled with a decrease in the total phosphorus concentration, indicating that the strain may perhaps possess mechanisms for degradation of glyphosate.

Foods and textiles industries

The *Trichoderma* lytic enzymes such as cellulases, hemicellulases, and pectinases are used as food additives in the production of fruit and vegetable juices and also to improve wine flavor and enhance fermentation, filtration, and quality of beer (Błaszczyk et al., 2014). Cellulases produced by *Trichoderma* are applied also in the textile industry to soften and condition the textiles as well as to produce high quality washing powders. In addition, these enzymes are used in the pulp and paper industry to modify fiber properties and to reduce lignin contents. In parallel, cellulases and hemicellulases produced by *T. reesei* are used in the production of bioethanol from farm wastes via degradation of substrates to simple sugars and then converted them to chemical intermediates such as ethanol (Błaszczyk et al., 2014). It must be mentioned that production of xylanase, cellulase and pectinases of *Trichoderma* account for 20% of the world enzyme market (Polizeli et al., 2005). In the food industry, xylanase enzymes help to break down polysaccharides in the dough of cookies, cakes, and aids in the digestibility of wheat by poultry by decreasing the viscosity of the feed (Harris and Ramalingam, 2010).

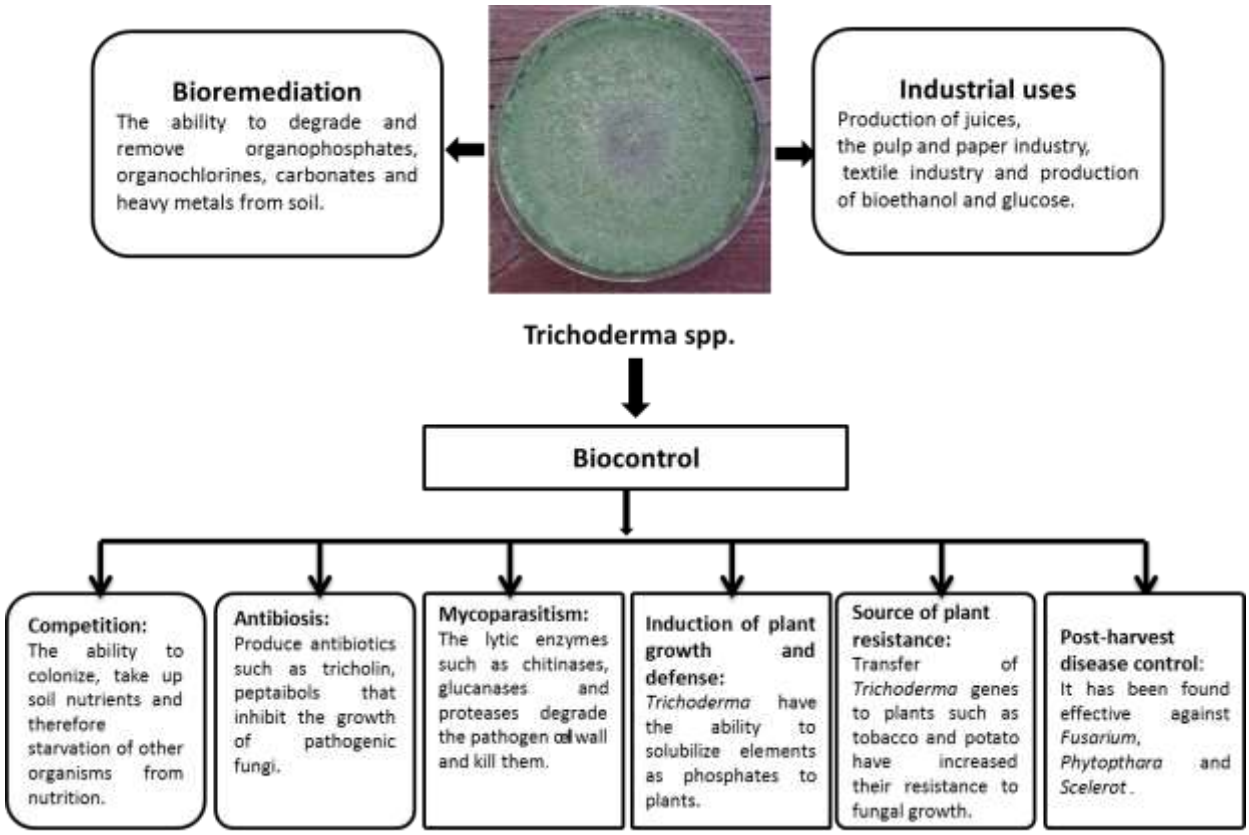

Figure 2. Different uses of *Trichoderma* in several fields.

Trichoderma genes as source of plant resistance

Trichoderma genes involved in pathogen cell wall degradation, such as chitinases and glucanases can be excellent sources for improving plant resistance against fungal pathogens. lorito et al. (1998) transferred the gene encoding a strongly antifungal endochitinase from the mycoparasitic fungus *T. harzianum* to tobacco and potato. High expression levels of the fungal gene were obtained in different plant tissues and this was linked with high resistance to the foliar pathogens *Alternaria alternata*, *A. solani*, *Botrytis cinerea*, and *R. solani*. Similarly, a chi gene from *T. asperellum*, designated *Tachi*, was cloned and transferred to soybean. Transgenic soybean plants with constitutive expression of *Tachi* showed increased resistance to *Sclerotinia sclerotiorum* compared to wild type plants. The overexpression of *Tachi* in soybean increased reactive oxygen species (ROS) level and each of peroxidase (POD) and catalase (SOD) activities. These results suggest that *Tachi* can improve disease resistance in plants by enhancing ROS accumulation and induction activities of ROS scavenging enzymes (Zhang et al., 2016). Moreover, Dana et al. (2006) generated transgenic tobacco (*Nicotiana tabacum*) lines that overexpress the endochitinases *CHIT33* and *CHIT42* from the mycoparasitic fungus *T. harzianum* and evaluated their tolerance to biotic and abiotic stress. The transformed plants with *CHIT33* and *CHIT42* exhibited broad resistance to fungal and bacterial pathogens, salinity, and heavy metals with no obvious effects on their growth. Furthermore, the endochitinase gene (Chit33-cDNA) of *T. atroviride* was overexpressed under the CaMV35S constitutive promoter in canola via *Agrobacterium tumefaciens* transformation. It was reported that lesion sizes of transgenic canola caused by *S. sclerotiorum* were significantly retarded when compared with non-transgenic canola plants (Solgi et al., 2015).

TOOLS FOR GENETIC IMPROVEMENT OF *TRICHODERMA*

Several genetic improvement trials are carried for maximizing the benefits of *Trichoderma* as biological control agents and in different industrial purposes. Recently, the genetic improvement of *Trichoderma* genus has entered a new era with the sequencing of *T. reesei*, *T. atroviride*, and *T. virens* genomes (Seidl and Seiboth, 2010; Mukherjee, 2011). The results indicated that the smallest genome size (34 Mb) was found in *T. reesei*,

while the largest genome (38.8 Mb) was recorded for *T. virens* (Mukherjee, 2011). Here, some of the genetic tools used for improving the biocontrol activity of *Trichoderma* against soil borne pathogens were highlighted.

Mutation

Mutagenesis is an excellent tools for developing *Trichoderma* mutants with enhanced secreted enzymes yields as compared to the parent strains (Seidl and Seiboth, 2010; Singh et al., 2016). Khandoker et al. (2013) employed ultraviolet (UV) irradiation and Ethidium bromide (EtBr) treatments to improve the production of cellulases from *T. viride*. The mutants of *T. viride* treated with UV and EtBr gave the highest cellulase activity with 11.28 and 14.61 U/ml, respectively as compared to 5.52 U/ml for the parent strain. In addition, Abbasi et al. (2014) used gamma rays for obtaining mutants of *T. harzianum* with maximum growth inhibition against *Macrophomina phaseolina*. It was found that the charcoal rot disease of melon reduced with 28% in the treated plants with *Trichoderma* mutants as compared to control. Moreover, N_methyl-N_nitro-N_guanidine (NTG) was used as mutagen for enhancing the antagonistic abilities of *T. harzianum*-1432 and *T. atroviride* against *Sclerotium rolfsii*, the causal agent of chickpea collar rot (Rashmi et al., 2016). The antagonistic capability of *T. harzianum* against *M. phaseolina*, *A. flavus* and *A. parasiticus* as pathogens was improved after exposure to UV-irradiation. Some mutants of *T. harzianum* released higher level of lytic enzymes, chitinases and cellulases (Singh et al., 2010; Patil and lunge, 2012; Walunj and John, 2015). Finally, γ-radiation induced mutants of *T. viride* with high ability to restrict *M. phaseolina* (Baharvand et al., 2014).

Protoplast fusion

Protoplast fusion is an important improvement tool for developing hybrid strains and improving the biocontrol potential of *Trichoderma* where the sexual cycle is difficult (Kowsari et al., 2014). Kowsari et al. (2014a) obtained some of *T. harizianum* fusants that expressed 1.5 fold of *chit42* transcript and exhibited a higher growth inhibition rate against *R. solani* than the parent strain. Moreover, Balasubramanian et al. (2012) carried out protoplast fusion between *T. harzianum* and *T. viride*. The *Trichoderma* HF9 fusant exhibited 3, 2.5 and 1.5-fold increase of total chitinase, specific chitinase and protein, respectively as compared with parent strains. Similarly, Mohamed and Haggag (2010) obtained fusants between *T. koningii* and *T. reesei* and most of these fusants showed superiority in their antagonistic activity against fungal pathogens which cause root-rot and damping-off diseases than their parental strains. Furthermore, Prabavathy et al. (2006) carried out self-fusion of *T. harzianum* strain PTh18 protoplasts and among the fusants, the strain SFTh8 produced maximum chitinase with a two-fold increase as compared to the parent strain. All the self-fusants exhibited high antagonistic activity against *R. solani* than the parent.

Genetic engineering

The huge progress in DNA sequencing techniques and comparative genomics analysis of different organisms has provided large lists of genes and their functions. Modification of *Trichoderma* genome by directly manipulating the DNA sequence of specific genes is considered modern and efficient tool to obtain strains with desired traits. Giczey et al. (1998) cloned a 42-kDa endochitinase encoding gene, *Tham-ch* from *T. hamatum* strain Tam-61. The *Tham-ch* with its own regulatory sequences was reintroduced into the host strain. Most of the transformants expressed higher levels of chitinase activity with 5-fold in comparison with the wild-type recipient strain. Moreover, Mendoza et al. (2003) cloned a mitogen-activated protein kinase encoding gene, *tvk1*, from *T. virens* and examined its role during the mycoparasitism, conidiation, and biocontrol in tvk1 null mutants. The null mutants displayed an increased protein secretion of lytic enzymes in culture supernatant compared to the wild type. Consistently, biocontrol assays demonstrated that the null mutants were considerably more effective in disease control than the wild-type strain or a chemical fungicide. These data suggest that Tvk1 acts as a negative modulator during host sensing and sporulation in *T. virens*.

A chimeric chitinase with improved enzyme activity was produced by fusing a ChBD from *T. atroviride* chitinase 18 to 10 with Chit42 (Kowsari et al., 2014b). The Chit42-ChBD transformants showed higher antifungal activity towards seven phytopathogenic fungal species suggesting that ChBD provides a strong binding capacity to insoluble chitin. In parallel, *T. atroviride* was transformed with *Aspergillus niger* glucose oxidase-encoding gene, *goxA*, under a homologous chitinase (*nag1*) promoter (Brunner et al., 2005). The transgenic strain was more quickly overgrown and lysed the plant pathogens *R. solani* and *P. ultimum* than control.

CONCLUSION

The genus of *Trichoderma* are widely used in agriculture and industry sectors due to its production of important lytic enzymes such as chitinases, glucanases, and proteases. Several genetic improvement trials are carried out for maximizing the role of *Trichoderma* as biological control agents via mutation, protoplast fusion and genetic transformation. Additional efforts must be done for

isolation of new strains with high antagonistic abilities and more violent against soil borne plant pathogens as a safe alternative than pesticides.

CONFLICT OF INTEREST

The author has not declared any conflict of interest.

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Overexpression of key enzymes of the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway for improving squalene production in *Escherichia coli*

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2-C-Methyl-D-erythritol-4-phosphate (MEP) pathway has been extensively employed for terpenoids biosynthesis in *Escherichia coli*. In this study, to obtain key-enzymes of MEP pathway for squalene production, overexpression of different combination of MEP pathway genes were compared. Squalene production in strain YSS12 with overexpressed *dxs*, *idi* and *ispA* of MEP pathway from *E. coli* was improved by 71-fold when compared with strain YSS3 which only contained double copy SQS. Analysis of transcriptional levels of MEP pathway genes in engineering strains showed that different squalene production can be attributed to changed transcriptional levels of co-overexpressed genes *dxs*, *idi*, *ispG* and *ispA* in engineering strains. Furthermore, different *E. coli* expression hosts were compared for squalene production, among which BL21(DE3) was the best squalene producer. These results illustrate that *dxs, idi* and ispA of the MEP pathway from *E. coli* were key-enzymes for squalene production in *E. coli*. These key-enzymes of MEP pathway could also be applied to other terpenoids production in *E. coli*.

Key words: Squalene, key-enzyme, 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway, *Escherichia coli*.

INTRODUCTION

Squalene is a triterpene with a unique 30-carbon, polyunsaturated hydrocarbon and has a variety of pharmacological activities such as reduction of serum cholesterol levels (Hien et al., 2017), anticancer (Kotelevets et al., 2017), modulating fatty acid metabolism (Kumar et al., 2016), and is extensively used in the functional food, cosmetic and pharmaceutical industries.

Naturally, squalene is derived from two universal precursors, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which are synthesized via the 2-C-methyl-D-erythritol-4-phosphate (MEP), or mevalonate (MVA) pathway (Banerjee and Sharkey, 2014). IPP and DMAPP are condensed to form geranyl diphosphate (GPP) by FPP synthase, and subsequently condensed with another IPP to produce farnesyl diphosphate (FPP). Finally, squalene is biosynthesized by a NADPH-mediated reaction catalyzed by squalene synthase (SQS) using FPP as the substrate (Ghimire et al., 2016) (Figure 1). MEP pathway is a

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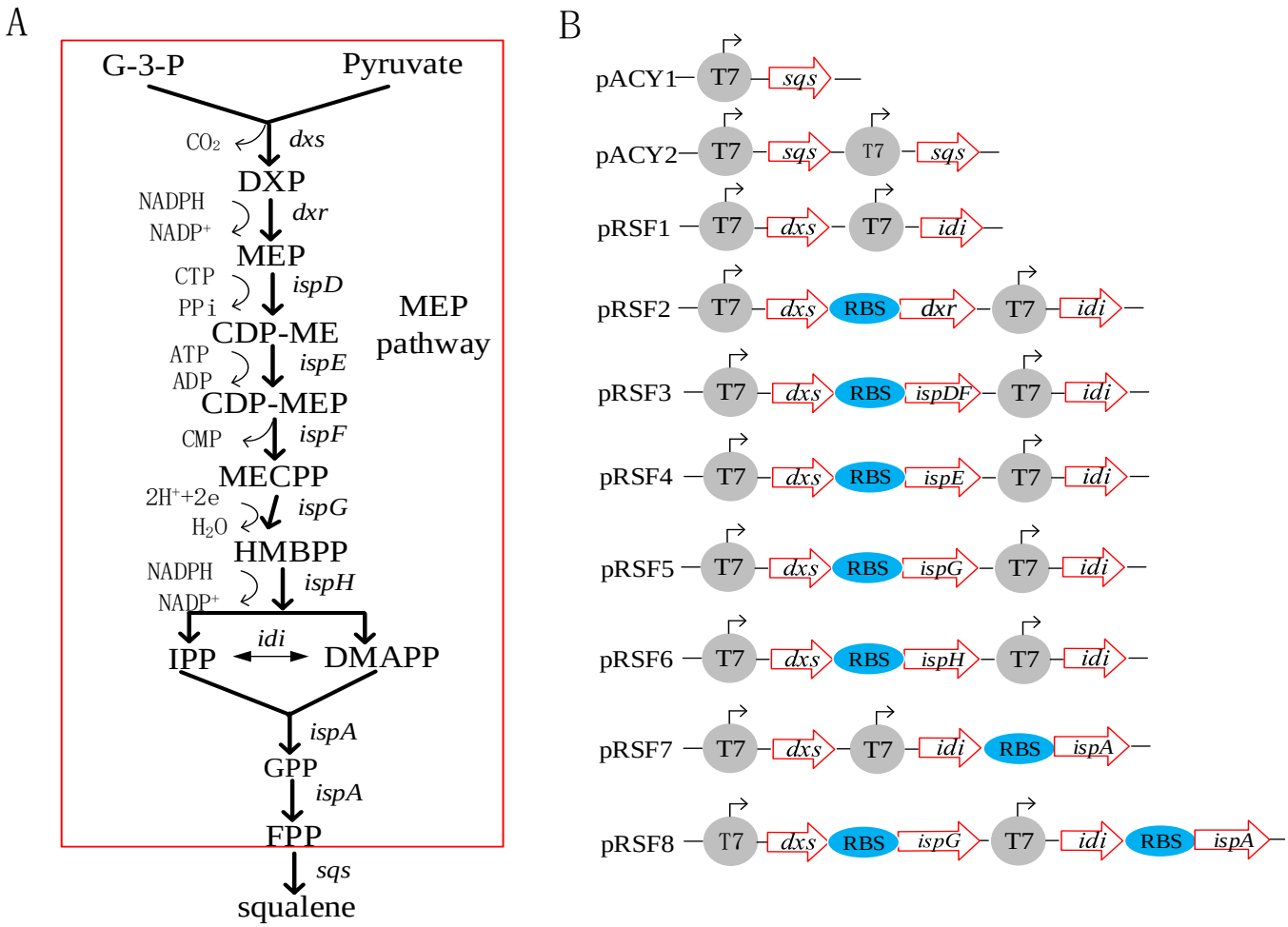

Figure 1. Biosynthesis of squalene in engineered *E. coli*. **A.** The native MEP pathway in *E. coli* consists of twelve metabolisms and nine enzymes: G-3-P, glyceraldehyde-3-phosphate; Pyruvate; DXP, 1-deoxy-D-xylulose-5-phosphate; MEP, 2C-methyl-D-erythritol-4-phosphate; CDP-ME, 4-diphospho-cytidyl-2C-methyl-D-erythritol; CDP-MEP, 4-diphosphocytidyl-2C-methyl-D-ery-thritol-2-phosphate; MECPP, 2C-methyl-D-erythritol 2,4-cyclodiphosphate; HMBPP, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate. **B.** Plasmid constructs used for squalene production in *E. coli.* T7, T7 promoter; RBS, ribosome binding site.

natural metabolic pathway and only produce trace amount of IPP and DMAPP in *Escherichia coli* that are precursors of all terpenoids. It consists of ten reactions catalyzed by nine enzymes (Figure 1a). Overexpression of MEP pathway genes were proven to be an effective method for increasing metabolic flux to IPP and DMAPP for terpenoid production in *Escherichia coli* (Jiang et al., 2012). DXS and IDI have been reported as the key-enzymes in the MEP pathway for increasing terpenoid production in *E. coli* (Yuan et al., 2006; Zhao et al., 2013) and squalene production (Ghimire et al., 2009). Overexpression of genes *dxr* (Lv et al., 2016), *ispDF* (Ajikumar et al., 2010; Yuan et al., 2006), *ispG* (Liu et al., 2014) and *ispA* (Han et al., 2016) were able to enhance terpenoids production. On the contrary, other studies have shown that overexpression of *dxr* coupled with *dxs* produced a similar isoprene level when compared with the *dxs* overproduction strain (Xue and Ahring, 2011), and overexpression of *ispDF* together with *dxs* and *idi* resulted in decrease in terpenoids production (Zhou et al., 2012). Meanwhile, genes *ispH*, *ispE* and *ispA* have not been overexpressed in combination with *dxs* and *idi* genes of MEP pathway in the production of terpenoids in *E. coli*. Based on the above studies, it is believed that in addition to the *dxs* and *idi* genes, other genes may be very important in the MEP pathway for squalene production in *E. coli*.

In this study, in order to clarify key-enzymes of MEP pathway for squalene production in *E. coli*, squalene biosynthetic pathway was constructed by overexpressing SQS in *E. coli*. The authors also introduced different gene combinations of MEP pathway in the squalene producer to identify key-enzymes for squalene production and to study the correlation between the transcriptional levels of

Table 1. Plasmids and strains used in this study.

Name	Relevant characteristics	Source
Plasmids		
pACYCDuet-1	P15A origin; Cm^R; P_{T7}	Novagen
pRSFDuet-1	RSF origin; Kn^R; P_{T7}	Novagen
pACY1	P15A origin; Cm^R; P_{T7}::*sqs*	This work
pACY2	P15A origin; Cm^R; P_{T7}::*sqs*; P_{T7}::*sqs*	This work
pRSF1	RSF origin; Kn^R; P_{T7}::*dxs*; P_{T7}::*idi*	This work
pRSF2	RSF origin; Kn^R; P_{T7}::*dxs-dxr*; P_{T7}::*idi*	This work
pRSF3	RSF origin; Kn^R; P_{T7}::*dxs-ispDF*; P_{T7}::*idi*	This work
pRSF4	RSF origin; Kn^R; P_{T7}::*dxs-ispE*; P_{T7}::*idi*	This work
pRSF5	RSF origin; Kn^R; P_{T7}::*dxs-ispG*; P_{T7}::*idi*	This work
pRSF6	RSF origin; Kn^R; P_{T7}::*dxs-ispH*; P_{T7}::*idi*	This work
pRSF7	RSF origin; Kn^R; P_{T7}::*dxs*; P_{T7}::*idi-ispA*	This work
pRSF8	RSF origin; Kn^R; P_{T7}::*dxs-ispG*; P_{T7}::*idi-ispA*	This work
Strains		
Yarrowia lipolytica (ATCC 20362)	WT	ATCC
E.coli DH5α	F-φ80 *lac* ZΔM15 Δ(*lac*ZYA-*arg* F) U169 *end* A1 *rec*A1 *hsd*R17(r_k^-,m_k^+) *sup*E44λ- *thi* -1 *gyr*A96 *rel*A1 *pho*A	Weidi
E.coli K12 MG1655	F^-, λ^-, ilvG$^-$, rfb-50, rph1	Our laboratory
BL21(DE3)	F^- *omp*T *hsd*S_B (r_B^- m_B^-) *gal dcm* (DE3)	Weidi
BL21 Star (DE3)	F^- *omp*T *hsd*S_B(r_B^- m_B^-) *gal dcm rne*131 (DE3)	Weidi
OverExpress C43(DE3)	F^-*omp*T *hsd*S_B (r_B^- m_B^-) *gal dcm* (DE3)	Weidi
Tuner(DE3)	F^- *omp*T *hsd*S_B(r_B^- m_B^-) *gal dcm lac*Y1 (DE3)	Weidi
YSS1	BL21(DE3)/ pACYCDuet-1	This work
YSS2	BL21(DE3)/ pACY1	This work
YSS3	BL21(DE3)/ pACY2	This work
YSS4	BL21(DE3)/ pACYCDuet-1, pRSFDuet-1	This work
YSS5	BL21(DE3)/ pACY2, pRSFDuet-1	This work
YSS6	BL21(DE3)/ pACY2, pRSF1	This work
YSS7	BL21(DE3)/ pACY2, pRSF2	This work
YSS8	BL21(DE3)/ pACY2, pRSF3	This work
YSS9	BL21(DE3)/ pACY2, pRSF4	This work
YSS10	BL21(DE3)/ pACY2, pRSF5	This work
YSS11	BL21(DE3)/ pACY2, pRSF6	This work
YSS12	BL21(DE3)/ pACY2, pRSF7	This work
YSS13	BL21(DE3)/ pACY2, pRSF8	This work
YSS14	BL21 Star(DE3)/ pACY2, pRSF7	This work
YSS15	OverExpressC43(DE3)/ pACY2, pRSF7	This work
YSS16	Tuner(DE3) / pACY2, pRSF7	This work

these genes and the yield of squalene. Finally, different *E. coli* strains were compared to determine the best host for squalene production.

MATERIALS AND METHODS

Bacterial strains and culture conditions

All strains used in this study are listed in Table 1. *E. coli* DH5α were Grown in LB medium at 37°C for plasmid construction. *E. coli* BL21(DE3), BL21 Star(DE3), OverExpress C43(DE3) and Tuner(DE3) (Shanghai Weidi Biotechnology Co., Ltd) were used to produce squalene. Recombinant strains were cultured in fermentation medium (Zheng et al., 2013) for squalene production. The cells were induced with 0.5 mM isopropyl β-D-thiogalactoside (IPTG) when OD_{600} reached 0.6 to 0.9 at 30°C and 180 rpm for 48 h.

Construction of recombinant plasmids

All plasmids used in this study are listed in Table 1 and all primers

used in this study are listed in Supplementary Table S1. Molecular biology protocols were carried out as described in the literature (Sambrook and Russell, 2001). DNA fragments were amplified by polymerase chain reaction (PCR) using PrimeSTAR® Max DNA polymerase (TaKaRa, Dalian, China) according to the manufacturer's instructions. All restriction enzymes and T4 DNA ligase were purchased from TakaRa (Dalian, China). DNA and plasmid extraction Kits were purchased from Shanghai Generay Biotech Co., Ltd. DNA sequencing and primers synthesis were provided by Shanghai Rui Di Biological Technology Co. Ltd.

The gene *sqs* was PCR amplified from *Yarrowia lipolytic* and MEP pathway genes as well as fragments such as *dxs-dxr*, *dxs-ispDF*, *dxs-ispE*, *dxs-ispG*, *dxs-ispH* and *idi-ispA* involved in this study were PCR or overlap PCR amplified *E. coli* K12 MG1655 genomic DNA using corresponding primer set (Table S1). DNA fragments and vectors were excised with restriction enzymes (Table S1) and ligated with T4 DNA ligase to create corresponding plasmids (Table 1 and Figure 1B).

Identification and quantification of squalene

After centrifugation at 8000 rpm for 5 min, 20 mL culture medium were mixed gently with 10 mL hexane by inverting the tube 5 times. After another centrifugation at 8000 rpm for 5 min, the hexane phase was collected. This extraction process was repeated one more time. Meanwhile, cell pellets were disrupted by ultrasonic in 3 mL acetone for three times. Hexane and acetone extracts were combined and evaporated under reduced pressure. The dry residue was dissolved in 300 µL of acetonitrile and filtered through a 0.25 µm filter prior to GC-MS or HPLC quantitative analysis.

The acetonitrile extracts (1 µL) were analyzed by GC-MS using a SHIMADZU GCMS-QP2010SE equipped with a Rxi-5ms (30 m × 0.25 mm × 0.25 µm) GC column. Compound separation was achieved with an injector temperature at 280°C, and a 30 min temperature gradient program for GC-separation starting at 200°C for 2 min followed by heating the column to 250°C at 20°C min^{-1} and a final constant hold at 250°C for 20.5 min. Mass detection was achieved with electric ionization using an EI scan mode with diagnostic ion monitored: *m/z* 69, 81 and 149. Squalene purchased from Aladdin®, China was used as standard.

For quantitative analysis of squalene, 20 µL acetonitrile extract was loaded onto an Agilent 1200 HPLC with UV detection at 210 nm. For chromatographic separation, a Waters SymmetryShield™ RP18 column (250 mm × 4.6 mm, 5 µm) was used. The mobile phase consisted of 2% water and 98% acetonitrile. The solvent flow rate was 1.0 mL/min and the column was held at 40°C during the separation. The peak area was converted into squalene concentration according to a standard curve plotted with a set of known concentrations of squalene.

Quantitative RT-PCR analysis

Cells cultured under fermentation condition were harvested by centrifugation at 12000 rpm and 4°C for 1 min. Total RNA was isolated using TRIzol Reagent (Sangon, Shanghai) following the manufacturer's instructions. RNA samples were treated with DNase I (TaKaRa, Dalian, China) for 30 min at 37°C. RNA was dissolved in 20 µL DEPC-H_2O and stored at -80°C. cDNA was reverse-transcribed with Prime Script™ RT reagent Kit (TaKaRa, Dalian, China) following the manufacturer's instruction and used as template for real-time PCR (qPCR). The primers used for qPCR are listed in Table S1. qPCR was carried out on a 7500 Real-Time PCR System (Applied Biosystems) using SYBR® Premix Ex Taq™ II kit (Tli RNaseH Plus) (TaKaRa, Dalian, China). The relative transcriptional levels were calculated by ΔΔCT method. The data were normalized using the *clpB* gene as an internal control. For each detected gene, the transcriptional level in control strain YSS4 was set to 1.

RESULTS

Establishment of squalene biosynthesis pathway in *E. coli*

Although *E. coli* can synthesize FPP which is a precursor of squalene by a native MEP pathway, it is unable to produce the squalene because of the absence of SQS. In order to establish squalene synthetic pathway in *E. coli* (Figure 1), the gene *sqs* from *Yarrowia lipolytica* was sub-cloned into pACYCDuet-1 and transformed into *E. coli* BL21 (DE3) for the first time to get engineering strain YSS2. GC/MS analysis of cell extraction confirmed the presence of squalene (Figure 2). No squalene was detected in the control strain YSS1 that only harbors the vector pACYCDuet-1 (Figure 2). These results suggested that SQS from *Y. lipolytica* can be used for the biosynthesis of squalene in *E. coli*.

Quantitative analysis of cell extraction by HPLC showed that YSS2 had a squalene yield of 0.072 mg/L at 48 h (Figure 3). The low production could be attributed to insufficient expression quantity of SQS and/or supply of precursors including IPP/DMAPP and FPP produced from native MEP pathway in *E. coli.*

The biosynthesis of squalene through overexpression of SQS in engineered *E. coli*

To increase the biosynthesis of terpenoid, it is also an effective way to overexpress the enzymes (Weaver et al., 2015). Trace amount of squalene produced by strain YSS2 may be because of low expression level of *sqs*. To enhance SQS concentration in *E. coli*, an extra copy of *sqs* was introduced into plasmid pACY1 resulting in the plasmid pACY2. The follow-up transformant YSS3 produced 0.15 mg/L squalene (Figure 3), which was approximately 2-fold of that produced by the strain YSS2. The results demonstrated that overexpression of *sqs* in *E. coli* is beneficial to the squalene production.

Finding key-enzymes of MEP pathway for squalene production in engineered *E. coli*

IPP and DMAPP are the universal precursors of all terpenoids in the living organisms (Martin et al., 2003). Increasing cellular metabolic flux towards IPP and DMAPP is an effective strategy to improve yield of terpenoids production (Leonard et al., 2010). In wild-type *E. coli*, MEP pathway is the unique origin for providing IPP and DMAPP. Genes *dxs* and *idi* of MEP pathway have been widely engineered to enhance the supply of IPP and DMAPP concentration in *E. coli* in order to increase synthesis of terpenoids (Zhao et al., 2013).

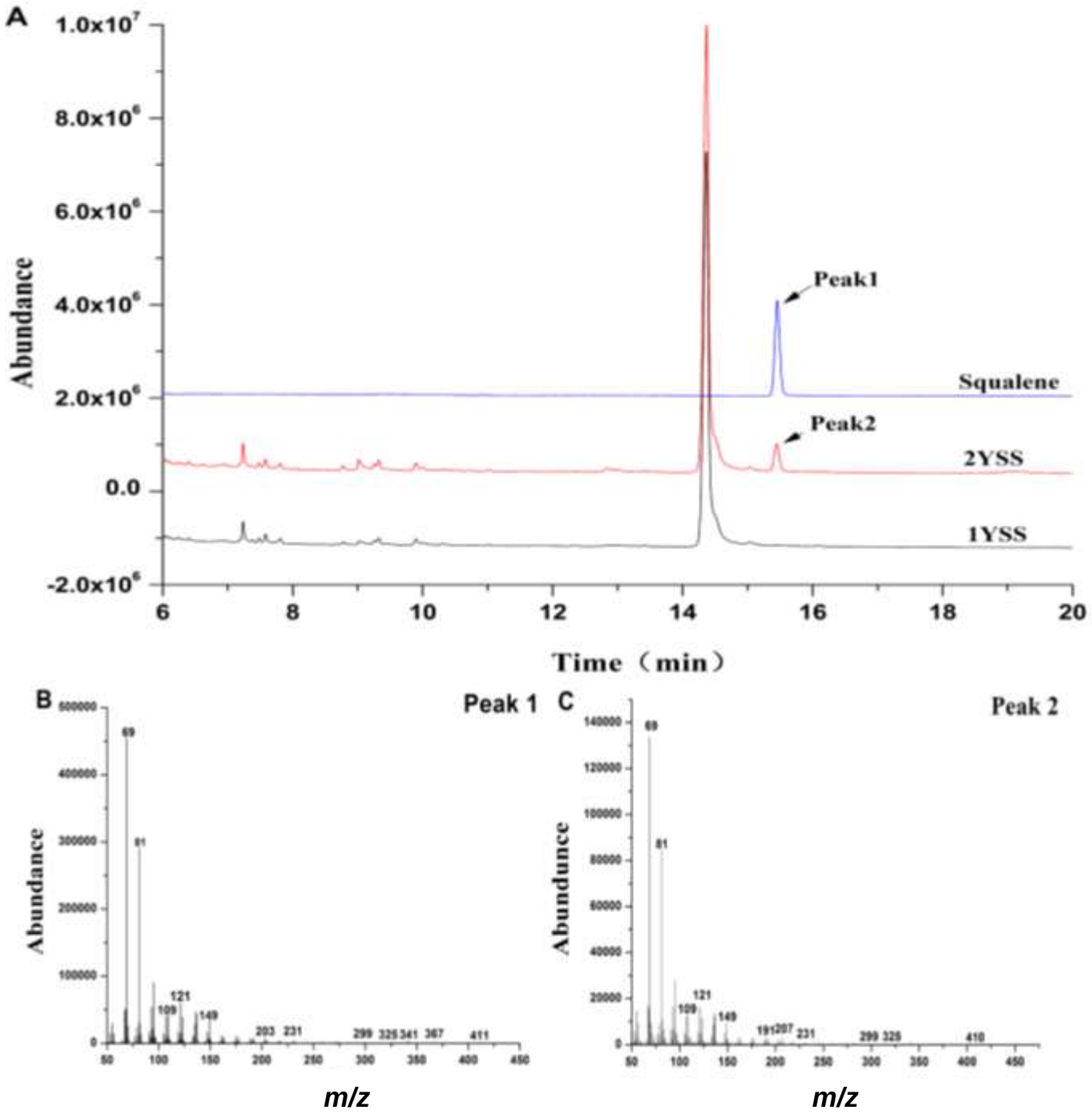

Figure 2. GC-MS identification and analysis of squalene from strain YSS2. **A.** Total ion chromatograms of squalene standard and the acetonitrile extracts from the strain YSS2 or control strain YSS1 containing an empty vector pACYCDute-1. **B.** Mass spectrum of squalene standard. **C.** Mass spectrum of acetonitrile extracts from the strain YSS2. Based on the relative retention time of total ion chromatograms and mass spectrum comparison with squalene standard, squalene production was identified.

Thus, *dxs* and *idi* genes were cloned and introduced into YSS3, resulting in strain YSS6. According to the HPLC analysis, strain YSS6 produced 3.68 mg/L squalene (Figure 4), a 24-fold higher than the strain YSS3. The results demonstrated that the DXS and IDI are key-enzymes for squalene production in *E. coli*.

To determine whether other enzymes of MEP pathway affect the biosynthesis of squalene, the authors cloned *dxr*, *ispDF*, *ispE*, *ispG*, *ispH* and *ispA* genes from *E. coli* K12 MG1655 genome to generate plasmids pRSF2,

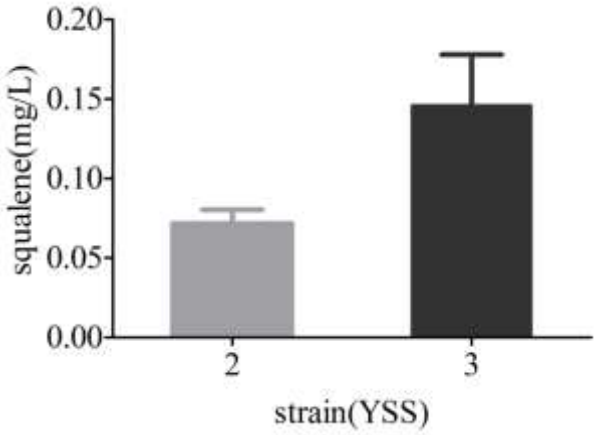

Figure 3. Squalene production in engineered *E. coli* BL21 (DE3). The experiment was performed in triplicate for each strain, and the error bars represent standard deviation.

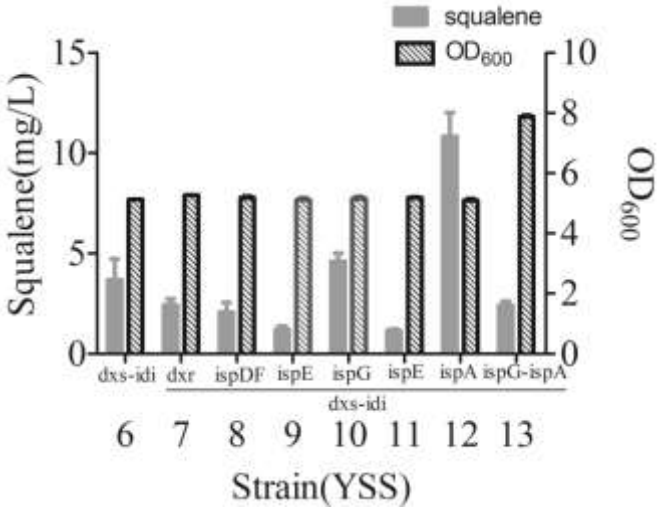

Figure 4. The effect of different combination of MEP pathway genes on biosynthesis of squalene in *E. coli*. The experiment was performed in triplicate for each strain, and the error bars represent standard deviation.

pRSF3, pRSF4, pRSF5, pRSF6 and pRSF7, respectively, as shown in Figure 1B. Co-transformation above plasmids respectively together with pACY2 resulted in six different strains. Figure 4 shows squalene production from these six different strains. Among these strain, YSS12 produced the highest squalene production at 10.83 mg/L, while YSS10 produced 4.60 mg/L, approximately 2-fold and 20% higher than that of YSS6, respectively. Squalene yields of strain YSS7, YSS8, YSS9 and YSS11 were 2.44, 2.07, 1.22 and 1.16 mg/L squalene, decreased by 34, 44, 67 and 68%, respectively, when compared with the YSS6. To further increase metabolic flux of the MEP pathway to squalene, plasmid pRSF8 was constructed by introducing *dxs*, *ispG*, *idi* and *ispA,* and together with plasmid pACY2, were co-transformed into BL21(DE3) to obtain strain YSS13. However, it only produced 1.6 mg/L squalene, decreased by 57, 65 and 85% when compared with YSS6, YSS10 and YSS12 (Figure 4). Comparing squalene production of all these strains, it showed that introducing *dxs*, *idi* and *ispA* could obtain highest squalene yield in YSS12 and the yield is approximately 71-fold when compared with the YSS3. Thus, DXS, IDI and IspA were considered to be key-enzyme of MEP pathway for squalene production in engineered *E. coli.*

Transcriptional levels analysis of MEP pathway genes in engineering *E. coli*

There are reports showing that metabolic imbalance by overexpression of certain genes in engineering metabolic pathway can lead to accumulation of toxic intermediates that produce inhibition of cell growth, metabolic flux overflow, gene transcription and enzymatic activity inhibition (Kim and Copley, 2012). To illuminate the relationship of overexpression of genes of MEP pathway with varying squalene production in engineering *E. coli*, the transcriptional levels of MEP pathway genes in these strains were measured by qPCR. As shown in Figure 5, when compared with the control strain YSS4, transcriptional levels of nine genes of MEP pathway were all weakly reduced in strain YSS5. This result illustrates that SQS/squalene could exert inhibitory effect on endogenous MEP pathway genes in engineering *E. coli.* The transcriptional level of overexpressed genes of MEP pathway in corresponding strains was significantly increased; however, other non-overexpressed genes have no remarkable changes. Compared with strain YSS6, transcriptional levels of *dxs* gene had about 36, 95 and 86% fold decrease in strains YSS7, 9, 11, and about 100, 0.5 and 7% increase in strain YSS8, 10 and 12. Transcriptional levels of *idi* gene had about 75, 193 and 143% increase in strains YSS8, 10 and 12 and about 19, 71 and 59% decrease in strains YSS7, 9 and 11. The transcriptional levels of *dxs*, *ispG*, *idi*, *ispA* genes in YSS13 were extremely reduced, as compared to YSS6, 10 and 12. Transcriptional levels of *dxs* and *idi* genes in YSS13 decreased by about 70 and 20% when compared with YSS6, and the transcriptional levels of *ispG* and *ispA* genes in YSS13 reduced by about 69 and 75% when compared with YSS10 and 12. These transcriptional results illustrated that overexpression of *dxr*, *ispE*, *ispG*, *ispH* and *ispA* and *ispG* genes of MEP pathway in strains 7, 9, 10, 11, 12 and 13 could influence transcriptional levels of co-overexpressed genes *dxs, idi, ispG* and *ispA,* which resulted in varying squalene production, except for the strain YSS8 with overexpression of *dxs*, *idi* and *ispDF* genes.

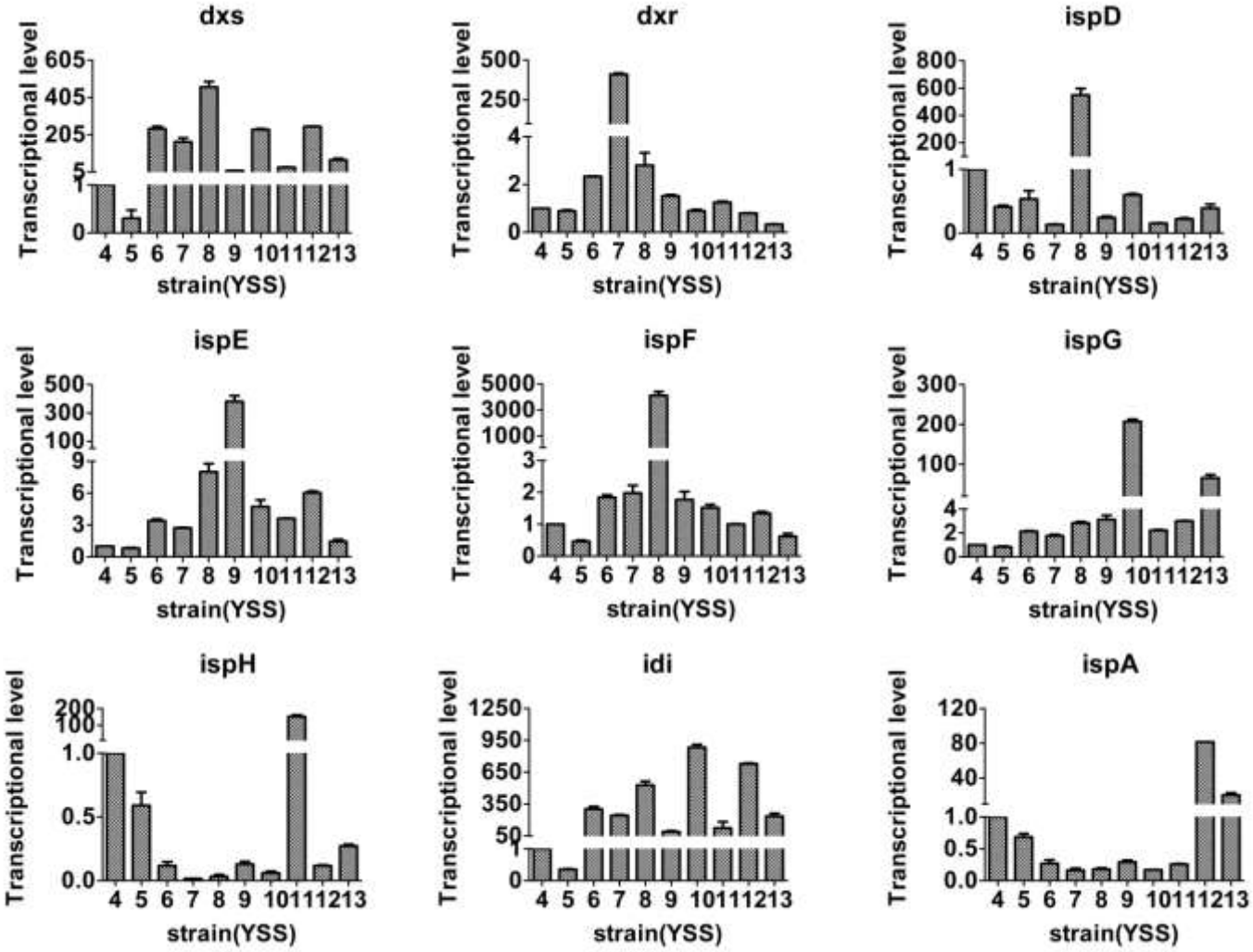

Figure 5. Relative transcriptional level analysis of MEP pathway genes in engineering strains. The relative abundance of mRNAs was standardized against the levels of *clpB* gene. Strain YSS4 is a control strain containing plasmid pACYDuet-1 and pRSFDuet-1, every gene of MEP pathway in strain YSS4 was set as 1. Vertical bars represent means ± SE (n = 3).

Comparison of squalene production in different *E. coli* strains

Metabolic pathway of terpenoids in *E. coli* could be obviously influenced by host strain with different genetic background and lead to different terpenoids production (Du et al., 2012). In order to choose an appropriate DE3 *E. coli* strain to maximize squalene production, pACY2 and pRSF7 plasmids were co-transformed into BL21 Star (DE3), OverExpress C43(DE3) and Tuner(DE3) strains, respectively to obtain strains YSS14, YSS15 and YSS16. After comparing these three strains with YSS12, it was found that YSS12 had the highest production of squalene (10.83 mg/L). YSS15 produced 9.28 mg/L squalene, YSS14 and YSS16 produced a much lower amount of squalene with 1.56 and 2.07 mg/L (Figure 6). Similar cell growth patterns were observed for all these strains. These results indicate that BL21 (DE3) was the most ideal strain for expression of the MEP pathway key enzyme DXS, IDI, IspA and SQS for squalene production.

DISCUSSION

In the process of terpenoid biosynthesis, introduction of exogenous MVA pathway into *E. coli* resulted in successful improvement for terpenoid production (Martin et al., 2003). However, previous study has demonstrated that native MEP pathway has a higher theoretical yield of terpenoid than MVA pathway in *E. coli* by genome-scale *in silico* modeling (Meng et al., 2011). Katabami et al. (2015) used truncated squalene synthases from human

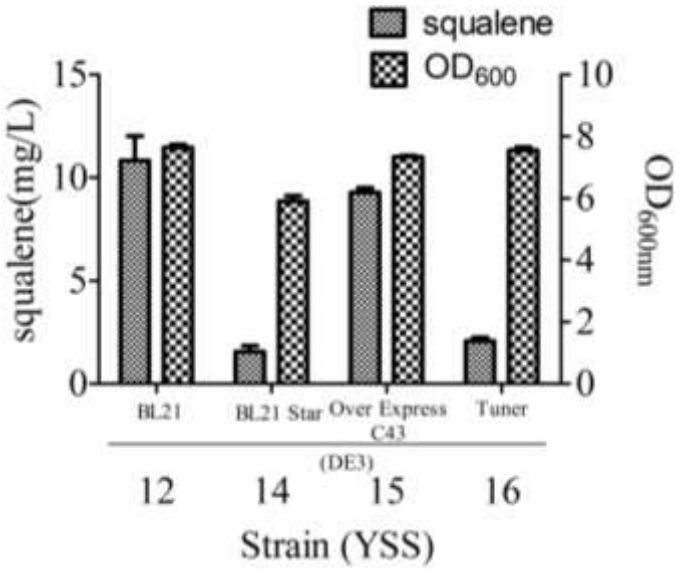

Figure 6. Squalene production in different BL21 (DE3) *E. coli* strains. These strains harbor plasmid pACY2 and pRSF8 under flask conditions. The experiment was performed in triplicate for each strain, and the error bars represent standard deviation.

(*hsqs*) in combination with MVA pathways to produce squalene up to 230 mg/L or 55 mg/g-DCW in flask culture, an approximately 55-fold increase as compared to *E. coli* harboring *hsqs* alone. In this study, in overexpression of key-enzymes genes *dxs*, *idi* and *ispA* of MEP pathway with double copy of SQS from *Y. lipolytica* in *E. coli* BL21(DE3), yield of squalene increased 71 folds, when compared with the strain that only harbor two copies of SQS. The result once again proved that the MEP pathway is superior to the MVA pathway for terpenoids biosynthesis in *E. coli*.

Presently, besides *dxs* and *idi*, other six genes of MEP pathway including *dxr* (Lv et al., 2016), *ispDF* (Ajikumar et al., 2010), *ispE* (Zhao et al., 2013) and *ispH* (Zhao et al., 2013) were explored for their potentials to increase terpenoids production. However, overexpression of *dxr*, *ispDF*, *ispH* and *ispE* together with *dxs* and *idi* decreased the squalene production when compared with the strain that overexpressed *dxs*, *idi* and double copy SQS, but enhanced squalene production when compared with the strain that only harbors two copies of SQS. These results illustrated that overexpression of *dxr*, *ispDF*, *ispH* and *ispE* coupled with *dxs* and *idi* did not substantially improve the yield of squalene but rather reduced DXS and IDI catalytic efficiency in the squalene production. Thus, it is suggested that overexpression of *dxr*, *ispDF*, *ispH* and *ispE,* respectively, together with *dxs* and *idi* are not helpful for terpenoids production in *E. coli*.

The transcriptional levels of *dxs* and *idi* genes in strain YSS8 were higher than that in strain YSS6, but squalene production was lower. Previous report (Zhou et al., 2012) showed that overexpression of *dxs*, *idi* and *ispDF* could lead to over-production and accumulation of MECPP in cell to outflow into the broth that is toxic to MEP pathway, which further decreased the lycopene production. The same situation may also appear in the current study where the outflow of MECPP produced by overexpression of genes *dxs*, *idi* and *ispDF* reduced toxic effect to MEP pathway, thus increased transcriptional levels of *dxs* and *idi* genes in strain YSS8. However, the outflow of MECPP also reduced the metabolic flux of the MEP pathway, and further decreased the production of squalene.

IspG is a valuable enzyme in MEP pathway for terpenoids production (Liu et al., 2014). In this study, similar result was observed by overexpression of *ispG* together with *dxs* and *idi* genes. However, squalene production was reduced by co-overexpression of dxs, ispG, idi and *ispA* in YSS13, and the transcriptional levels of overexpressed genes were also remarkably decreased when compared with strains YSS6, 10 and 12. This unexpected result may also be attributed to metabolic imbalance and toxic metabolites produced by overexpression of *ispG* together with *dxs*, *idi* and *ispA* in YSS13. Nevertheless, it is believed that IspG is an important enzyme and can be used to increase the production of squalene by balancing the flow of MEP pathway in future study.

IspA is also considered to be a key enzyme for terpenoids biosynthesis (Han et al., 2016), however *ispA* is not used extensively with MEP pathway genes but is widely used in the MVA pathway. Combination of *dxs*, *idi* and *ispA* can increase squalene production in YSS12 by up to 71-fold when compared with the strain that only harbors two copies of SQS. This can be ascribed to a more balanced and productive MEP pathway metabolic flux to squalene by overexpression of *ispA*. Therefore, it is suggested that overexpression of *dxs* and *idi* together with *ispA* is an effective strategy for terpenoids biosynthesis in *E. coli*.

Conclusions

In the engineering of *E. coli* that produced squalene of up to 0.15 mg/L, it was demonstrated that DXS, IDI and IspA of MEP pathway were key-enzymes for squalene production in BL21(DE3) by comparing the combinations of different MEP pathway genes. In brief, this work presented a promising strategy for the production of squalene in *E. coli,* and the key enzymes DXS, IDI and IspA could be used to effectively improve the production of other terpenoids in *E. coli*.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Table S1. Primers used in this study.

Name	Sequence(5'→3')
1T7YSS-F	CGGGATCCGATGGGAAAACTCATCGAACTG
1T7YSS-R	CCCAAGCTTCTAATCTCTCAGAGGAAACATCTTAGAGTCG
2T7YSS-F	GGAATTCCATATGGGAAAACTCATCGAACTGC
2T7YSS-R	GGGGTACCCTAATCTCTCAGAGGAAACATCTTAGAGTC
dxs-F	CGAGCTCGATGAGTTTTGATATTGCCAAATACCCGACCC
dxs-R	AACTGCAGTTATGCCAGCCAGGCCTTGATTTTG
idi-F	GGGGTACCATGCAAACGGAACACGTCATTTTATTGAATGC
idi-R	CCGCTCGAGTTATTTAAGCTGGGTAAATGCAG
dxsr-R	GTGAGTTGCTTCATGGTATATCTCCTTTTATGCCAGCCAGGCCTTG
dxr-F	CTGGCTGGCATAAAAGGAGATATACCATGAAGCAACTCACCATTC
dxr-R	AACTGCAGTCAGCTTGCGAGACGCATCACCTCTTTTC
dxsF-R	GAGTGGTTGCCATGGTATATCTCCTTTTATGCCAGCCAGGCCTTGAT
ispDF-F	CTGGCTGGCATAAAAGGAGATATACCATGGCAACCACTCATTTG
ispDF-R	AACTGCAGTCATTTTGTTGCCTTAATGAGTAGCGCCACCG
dxsE-R	CTGTGTCCGCATGGTATATCTCCTTTTATGCCAGCCAGGCCTTGAT
ispE-F	CTGGCTGGCATAAAAGGAGATATACCATGCGGACACAGTGGC
ispE-R	AACTGCAGTTAAAGCATGGCTCTGTGCAATGGGGAAAG
dxsG-R	GGTTATGCATGGTATATCTCCTTTTATGCCAGCCAGGCCTTGATTTTG
ispG-F	CTGGCTGGCATAAAAGGAGATATACCATGCATAACCAGGCTCC
ispG-R	AACTGCAGTTATTTTTCAACCTGCTGAACGTCAATTCGAC
dxsH-R	GGATCTGCATGGTATATCTCCTTTTATGCCAGCCAGGCCTTGATTTTG
ispH-F	CCTGGCTGGCATAAAAGGAGATATACCATGCAGATCCTGTTGGCCAAC
ispH-R	AACTGCAGTTAATCGACTTCACGAATATCGACACGCAGCTC
idiA-R	CGGAAAGTCCATGGTATATCTCCTTTTATTTAAGCTGGGTAAATGCAG
ispA-F	CAGCTTAAATAAAAGGAGATATACCATGGACTTTCCGCAGCAACTC
ispA-R	CCGCTCGAGTTATTTATTACGCTGGATGATGTAGTCCGC
qdxs-F	ACTCCACCCAGGAGTTACGACTGTT
qdxs-R	ATAGTGCAGCGCCACGGTCA
qdxr-F	TGTTTATGGACGCCGTAAAG
qdxr-R	CACCAGACCCGGTAAGTAAA
qispD-F	ACCATTCTTGAACACTCGGTGC
qispD-R	CAGACCTGCCAGCACGGAAT
qispE-F	GGCCCTCTCCGGCAAAACTTAAT
qispE-R	CAACGGGCGTTAACAGACGA
qispF-F	ATGCGAATTGGACACGGTTTTG
qispF-R	GCCAAGCAATGCATCGGTCA
qispG-F	ATGCATAACCAGGCTCCAAT
qispG-R	AGCGCCTTGATTTGATTGAC
qispH-F	GATCCTGATTTTCTCCGCAC
qispH-R	AGAATAGATTCTTCGCCACG
qidi-F	ATGCAAACGGAACACGTCAT
qidi-R	CGCGGCGGGTAACTAATAAT
qispA-F	ATGGACTTTCCGCAGCAACT
qispA-R	AAACCAGGAAAGGTCGCAGG
clpB-F	ATGCGTCTGGATCGTCTTAC
clpB-R	GCCAGCGGATGTTAATAAAG

A homologous arm for overlap PCR is indicated by dotted underline, RBS sequence are virtual underline, restriction sites are underlined.

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Full Length Research Paper

Genetic and population diversity of bacuri (*Platonia insignis* Mart.) in Chapada Limpa extractive reserve, Maranhão State, Brazil

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Landscape studies illustrate how the structure and configuration of ecological/agroecological zones influence the genetic diversity of a species. Thus, the aim of the present study is to assess how landscape structure and conservation influence the genetic diversity indices of *Platonia insignis* Mart. (bacuri) in Chapada Limpa Extractive Reserve, Chapadinha County, Maranhão State, Brazil. The study was carried out in an extractive reserve, whose surroundings are mapped for land use and occupation, as well as for the collection of bacuri biological materials used in genetic analysis with ISSR markers. The study shows there is prevalence of good preservation state and secondary vegetation, as well as a well-preserved Cerrado area, *lato sensu*. Genetic differences were higher within populations (85.2%) than between populations (14.7%). According to the genetic diversity index, there was high genetic diversity and heterozygous allele richness. This may ensure population viability. Based on the present results, it is possible to say that the genetic differentiation of *bacuri* may have suffered direct consequences from the intensive wildfire events and/or from primary vegetation reduction.

Key words: *Platonia insignis*, mapping, landscape, genetic diversity.

INTRODUCTION

Bacuri (*Platonia insignis* Mart.), belonging to the division Magnoliophyta, Class Magnoliopsida, order Malpighiales and family Clusiaceae, is the only species of the genus *Platonia* (Cavalcante, 1996). It is a fruitful tree distributed in Maranhense Amazon Region (Souza et al., 2000) and natural to the Amazon (Cavalcante, 1996; Moraes et al., 1994). It is also naturally found in other Brazilian Amazonian states (Loureiro et al., 1979; Cavalcante, 1996). It stands out for its economic relevance in the Amazonian region due to its management and sustainable development (Alvarez and Potiguara, 2013).

Bacuri trees are found in areas with intense agricultural activities in Maranhão State, where deforestation and wildfire events are common. Consequently, the number of plants has been rapidly reducing, a fact that leads to decreased genetic variability (Carvalho et al., 2009). The

aforementioned species went from an unimportant fruit tree to a timber tree of interest. Besides, its fruit became valuable. Leafy Bacuri trees were cut down over centuries for wood extraction, for use in the construction industry, and for mainly artisanal shipbuilding in the Amazon (Homma et al., 2007). Therefore, gradual Cerrado vegetation losses and bacuri clearing led to great damage to the species genetic diversity.

Inter Simple Sequence Repeats (ISSR) (Reddy et al., 2002; Zietkiewicz et al., 1994) molecular marker was adopted in a microsatellite-based method. It does not require having prior knowledge about the genome or about the first clone design. Although ISSRs are dominant markers, they are useful in multiple loci analysis applied to a single reaction (Goulão and Oliveira, 2001). They have also been used to estimate the inter- and intra-specific genetic diversity of a wide variety of species (Zietkiewicz et al., 1994). ISSR molecular marker appears as a power tool for analyzing genetic diversity, as well as characterizing many species (Charters and Wilkinson, 2000; Isshiki et al., 2008).

Bacuri species diversity in forests is relevant to local communities that make their living on selling bacuri fruit pulp. Such scenario is one of the herein assessed study objects, since the aim of the present research is to investigate the conservation status of Cerrado areas wherein bacuri trees are distributed, as well as Cerrado surrounding areas and bacuri genetic diversity rates. Chapadinha County is surrounded by the Amazonian forest and large soybean plantations (Nogueira et al., 2012; Loch and Muniz, 2016). The conservation unit was launched in 2007 for sustainable use (Filho, 2014).

The diversity of bacuri species in the forests is of great importance to the local communities that live on selling the fruit pulp. The main goal of the present study is to set the genetic structure of bacuri trees and the species' distribution pattern at Chapada Limpa Extractive Reserve, Chapadinha County, East Maranhão State.

MATERIALS AND METHODS

P. insignis leaf tissue samples were collected. Samples from two subpopulations were divided into two groups (15 individuals per group) (Figure 1). The minimum space between individuals in each group was 15 m; ten leaves were collected from each individual. The samples were kept in silica gel until the DNA was extracted and the genetic analysis was performed. The research was authorized by Sisbio (no. 50329-1) and by the competent ICMBio-MA authority responsible for the extractive reserve.

Landscape analysis

Each collected individual was georeferenced for further landscape OpenLayers plug-in, at high resolution images (1×1 m) and 10% cloud coverage, on average, in all the study site fractions. The composition analysis. The extractive reserve mapping was performed based on the coordinates in the Quantum GIS Software 1.8 (QGis) (Sherman et al., 2011). This was done with the aid of the mapping scale was defined in the QGIS and set at 1:10.000. The mapping aimed at finding the land use and occupation state of bacuri trees in different areas, as well as in Chapada Limpa Extractive Reserve surroundings.

DNA extraction, polymerase chain reaction (PCR), ISSR markers and data analysis

Leaf tissue DNA samples were extracted from 30 *P. insignis* samples. Tissue maceration was performed in liquid nitrogen. The genomic DNA extraction was conducted based on the protocol by Doyle and Doyle (1987), using approximately 1 g of dry leaves from each sample. After the extraction procedure was over, the DNA was stained in ethidium bromide, and subjected to electrophoresis in 1% agarose gel. Next, DNA was diluted to 5 ng/µl, in 100 µl for further PCR, according to the method of Mullis and Faloona (1987) and Saiki et al. (1985).

Twelve ISSR marker loci were amplified and the ISSR primers were previously selected and developed according to Souza et al. (2013) (Table 1). The amplification products were separated through horizontal electrophoresis, in 1.5% agarose gel, for 90 min; subsequently, they were stained in ethidium bromide.

The PCR reactions were performed in final volume (20 µL) of a mixture containing the following components: PCR buffer [13.5 mM Tris-HCl (pH 8.3); 67.5 mM KCl; 2.0 mM $MgCl_2$], 200 µM of each one of the four deoxyribonucleotides (dATP, dCTP, dGTP and dTTP), 0.7 µM primers, 1 U Taq DNA polymerase, 5 ng of genomic DNA and water were amplified in Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystems), which was programmed as follows: 95°C for 10 min, 35 cycles [95°C for 1 min; 50 to 62°C (depending on the used primer) for 45 s and 72°C for 2 min]; and 72°C for 5 min.

The amplified products were designated as a single character, which had its presence represented by "1" and absence by "0". The ISSR markers were converted into a binominal matrix (0/1). Assumingly, all loci were dominant in all the performed analyses and in the Hardy-Weinberg equilibrium. The genetic relation between genotypes was estimated through the Jaccard's coefficient, which resulted in a similarity matrix. The compliance between the similarity matrix and the dendrogram was estimated through the cophenetic correlation coefficient (r), according to Sokal and Rohlf (1962). This dendrogram was found through the Unweighted Pair Group Method by using arithmetic means (UPGMA) to illustrate the relation between the two collected populations. All calculations were made in the PAST software, version 1.34 (Hammer et al., 2001).

The genetic structure of the population (Heterozygosity-He), as well as the genetic differentiation values of a single population or between populations (Fixation index-FST) (Weir and Cockerham, 1984) were analyzed in the Arlequin 3.11 software (Excoffier et al., 2005). The genetic bottleneck test was run in the Bottleneck 1.2 software (Cornuet and Luikart, 1997) in order to check whether there was recent or effective decrease in the populations through the infinite allele models (IAM) and step-wise mutation model (SMM). It is recommended to apply these analyses to ISSR marker

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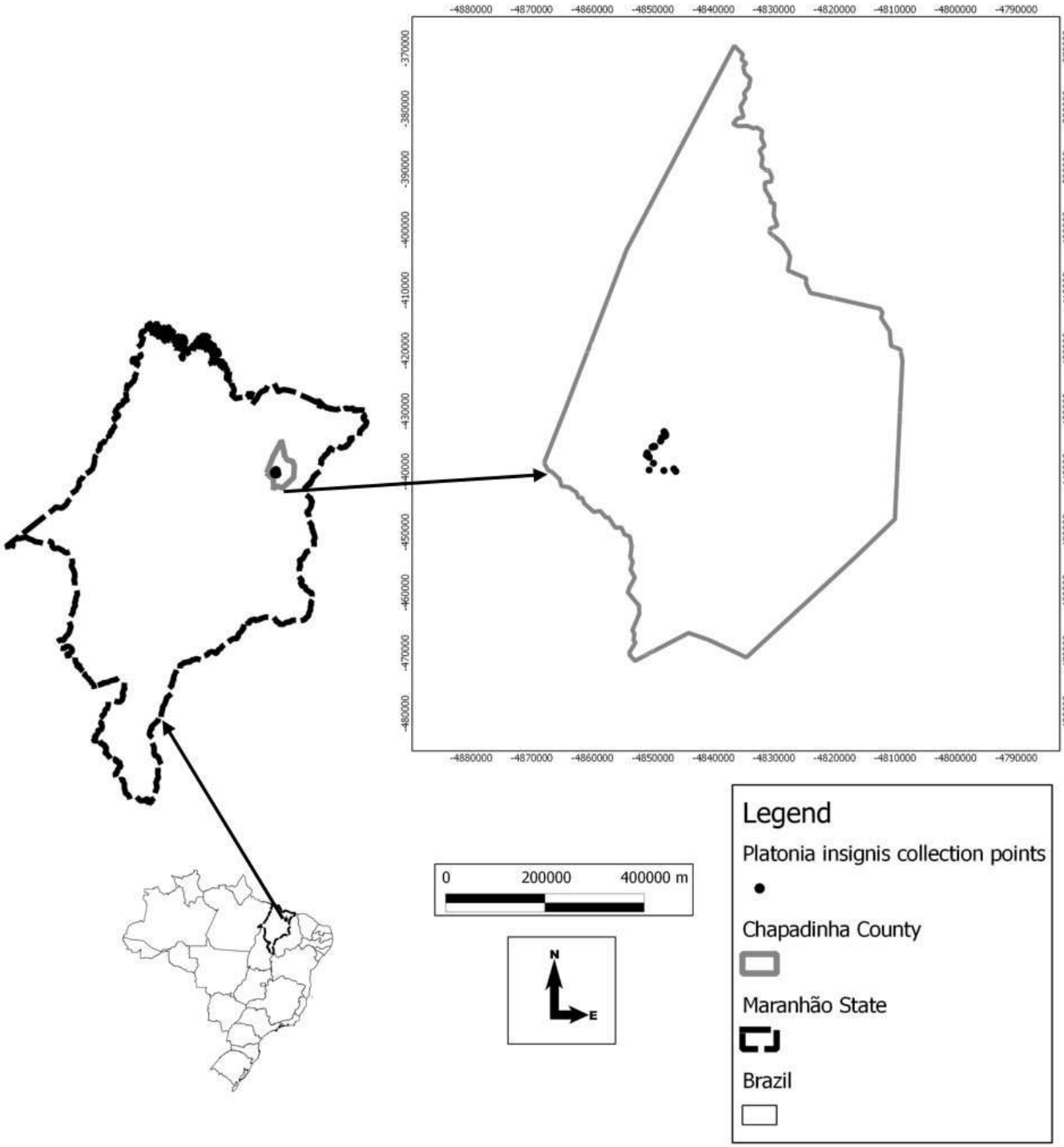

Figure 1. Chapadinha and Chapada Limpa Extractive Reserve location in the Maranhão State and *Platonia insignis* collection points.

data. He > Heq hypothesis was tested; wherein: He is the expected heterozygosity under the Hardy-Weinberg equilibrium; and Heq is the expected heterozygosity under balance of mutation-drift (P<0.0001).

The Shannon index analyzed through Fingerprint Analysis with Missing Data 1.31 (FAMD) was another diversity measure used in the present study (Schlüter, 2013). It measures the uncertainty degree of predictions made to the species of a randomly chosen individual belonging to a sample with S species and N individuals. The analysis of molecular variance (AMOVA) was also used to show the genetic diversity distribution within and between populations. The total genetic diversity recorded through the analysis was split into two distinct hierarchical levels, namely, differences between populations and between individuals within a

Table 1. List of the 15 primer loci, their sequences and amplification conditions tested in *Platonia insignis.*

Primer	Annealing temperature (ºC)	Sequence 5'-3'
UBC 807	53	(AG)7GT
UBC 808	54	(AG)8C
UBC 809	54	(AG)8G
UBC 810	53	(GA)8T
UBC 811	54	(GA)8C
UBC 817	53	(CA)8A
UBC 825	54	(AC)7A
UBC 826	59	(AC)8C
UBC 828	54	(TG)8A
UBC 829	52	(TG)8C
UBC 834	53	(AG)8YT
UBC 840	54	(GA)8YT

single population. AMOVA was performed according to Excoffier et al. (1992), with the aid of Arlequim and FAMD software.

All the steps in the bacuri genetic diversity analysis were carried out at Warwick Kerr (LabWick) Genetic and Biology Laboratory at Maranhão State Univesity.

RESULTS

Land use and occupation

The 67.3% prevalence of secondary vegetation was followed by 10.29% advanced vegetation and 9.21% initial vegetation (Figure 3). The Cerrado and bacuri forest area covered 4.59% of the map. This category can be classified as "Cerrado *stricto sensu*". The expectation was to have 9.21% of the entire mapped area to be covered with "advanced vegetation", which consisted of Cerrado areas, with high conservation degree. The "cultivation/agricultural area" accounted for only 0.08% of the total area (54,471 thousand hectares), although Maranhão State Cerrado is currently one of the most used biomes for extensive agriculture (Table 2).

Some of these land use and occupation formations were herein described. The analysis of classes followed the description by Ribeiro and Walter (2008) due to Resex landscape heterogeneity. The main formations found in the mapping process are as follows:

(1) Forest formations: This included vegetation types dominated by tree species of continuous canopy formation known as "advanced vegetation". It is possible to include the "secondary" and "shrub" vegetation classification in the phytophysiological category.
(2) Dry Forest: Formation type is defined as "initial vegetation" according to the present classification. The "pasture + shrub vegetation" and "pasture" (predominantly formed by low growing vegetation such as grass) classes were also included in the phytophysiological category.
(3) Cerrado *stricto sensu*: It is characterized by the presence of low, tilted, tortuous trees with irregular and twisted branches, often showing signs of burning.

Genetic analysis of the ISSR markers

AMOVA results (Table 3) of the genetic structure of the population showed high genetic diversity within populations (82.2%) and low diversity between populations (14.7%). The ϕST and FST indices were used to measure the genetic distance in the population; they were run in different software (FAMD and Arlequim). Significant genetic differences were found through the FAMD calculation: ϕST=0.17, P < 0.001; and FST= 0.147 (analyzed in the 3.5 Arlequim software). The genetic distance index calculated in the Arlequim was 0.17 for ϕST and 0.28 for Fst.

With regard to the genetic diversity index, heterozygosity variation (He) was found to be 0.133 to 0.533 (mean - 0.333) in population 1 and 0.133 to 0.514 (mean: 0.388) in population 2. The index showed variation from 0.163 to 0.393 in the comparison between the two populations. As for the arboreal species, this genetic diversity index set by Nei remains high; however, it can indicate small population difference and diversity loss in the short-term. When it comes to the bottleneck, He higher than Heq in most of the loci was recorded in both models (I.A.M. and S.M.M.), except for loci UBC 817 and UBC 825, which presented higher Heq values. The over heterozygosity in both models showed possible population decrease in the short-term (Table 4).

The Shannon H' diversity index calculated in the FAM software is commonly used in ecological studies to indicate species diversity per area. The Shannon index of the general population in the current study was 0.011 ≤ 0.106. This index was 0.030 ≤ 0.175 in population 1 and 0.041 ≤ 0.204 in population 2. Besides, the heterozygosity variation in the population ranged from

Table 2. Chapada Limpa Extractive Reserve land use and occupation classes and its surrounding areas, Chapadinha County, Maranhão State, Brazil.

Class	Hectares	%
Water	5.032,4	0.09
Growing area/agriculture	4.851,9	0.08
Babaçu (*Attalea speciosa*) + shrub vegetation	14.833,10	2.72
Cerrado/Bacuri forest	24.964,20	4.59
Local roads (dirty or not)	10.177,79	1.96
Pasture	74.290	0.13
Pasture + shrub vegetation	9.630,21	1.78
Residences	4.076,9	0.07
Exposed soil	15.499,9	0.28
Shrub vegetation	81,572.6	1.5
Advanced vegetation	56,043,11	10.29
Initial vegetation	50,205,07	9.21
Secondary vegetation	36.701,580	67.3
Total	54.471.555	-

Table 3. AMOVA values for *Platonia insignis* populations at Extractive Reserve Chapada Limpa, Chapadinha County, Maranhão State.

Variation source	Square sources	Variance components	Variation rate
Between populations	0.800	0.37	14.77273
Within populations	60.667	2.16667	85.22727
Total	68.467	2.54222	-
FST	0.14773	-	-
ϕST	0.17192	-	-

0.133 to 0.533 in population 1, but from 0.133 to 0.514 in population 2. However, the index ranged from 0.163 to 0.393 in the comparison between the two populations. The similar Jacard coefficient values calculated in the distance matrix ranged from 0.818 to 0.111, thus indicating high and low correlations between individuals. The dendrogram showed 10 small clusters demonstrating clusters comprising individuals belonging to populations 1 and 2 in separate groups, as well as clusters comprising individuals belonging to both populations in a single group (Figure 2).

DISCUSSION

Land use and occupation

Well preserved natural classes were found in Chapada Limpa Extractive Reserve; however, the frequent wildfire events are an eminent danger for the region. Morelli et al. (2009) emphasized that the use and occupation restrictions promoted by protection area (PA) implementation do not prevent wildfire to occur within these areas. Gerude (2013) stated that, despite the protective nature of these spaces, protected areas (UCSf, USEs and TIs) account for 19.5% of the wildfire outbreaks in Maranhão State. According to Gerude, there were 29 annual wildfire outbreaks in the reserve, between 2008 and 2012. Primary and secondary vegetation replacement has become a major factor, mainly in areas of agricultural vocation of mechanized or family-based nature. Areas holding regenerating vegetation (secondary vegetation) were found in the mapping performed by Sano et al. (2008), mainly, in transition regions of the Amazonian biome; the region mapped in the present study is located right in this ecotone area.

Landscape heterogeneity at Extractive Reserve Chapada Limpa presents different phytophysiognomies, including the “Cerrado *lato sensu* class”, as well as secondary vegetation and coconut groves. According to Conceição and Castro (2009), Maranhão State has approximately 10.000.000 hectares of Cerrado area, and it represents 30% of its territorial extension, and 5% of the Brazilian total Cerrado area. The area has low-fertility and great soil variation, as well as high acidity; it has little water content. It is predominantly mechanized due to agricultural purposes.

Table 4. Bottleneck Test and Nei's diversity indices for *Platonia insignis* populations at Extractive Reserve Chapada Limpa, Chapadinha County, Maranhão State.

Locus	Bottleneck Test			Nei's diversity indices				
	He	I.A.M.	S.M.M.	He	He	Mean	s.d.	Tot. Het.
		Heq	Heq	Pop 1	Pop 2			
UBC 807	0.480	0.254	0.286	0.533	0.342	0.438	0.134	0.480
UBC 808	0.370	0.249	0.293	0.247	0.476	0.161	0.161	0.370
UBC 809	0.515	0.245	0.287	0.533	0.514	0.523	0.013	0.514
UBC 810	0.517	0.246	0.285	0.533	0.533	0.533	0.000	0.517
UBC 811	0.370	0.235	0.283	0.476	0.247	0.361	0.161	0.370
UBC 817	0.067	0.250	0.289	0.000	0.133	0.066	0.094	0.066
UBC 825	0.067	0.248	0.284	0.133	0.000	0.066	0.094	0.066
UBC 826	0.460	0.245	0.284	0.000	0.476	0.238	0.336	0.459
UBC 828	0.480	0.243	0.282	0.247	0.514	0.380	0.188	0.480
UBC 829	0.370	0.251	0.285	0.247	0.476	0.361	0.161	0.370
UBC 834	0.508	0.230	0.287	0.514	0.419	0.466	0.067	0.508
UBC 840	0.517	0.249	0.292	0.533	0.533	0.533	0.000	0.517
Mean	0.393	0.245	0.286	0.333	0.388	0.361	0.039	0.393

He, heterozygosity variation; Heq, expected heterozygosity; IAM, infinite allele models; SMM, step-wise mutation model.

Genetic analysis of ISSR markers

There are different ways to quantify genetic diversity, namely, number of alleles per locus, polymorphic loci percentage; expected heterozygosity (Nei's diversity indices), which is the proportion of heterozygotes expected for a population in Hardy-Weinberg equilibrium (Freeland, 2005) and Shannon index. Souza (2011) recorded FST difference 0.28 in his studies about bacuri populations in Maranhão and Piauí states. Pontes et al. (2015) found ϕST 0.05 (P≥0.001), 6% variation between progenies and 94% (AMOVA) genetic variation within progenies of bacuri tree populations collected in Marajó Island, Pará State. The Nei (1973) gene diversity index represents population hererozygosity (He); it generates values between 0 and 5 for each locus. Souza (2011) compared results of Nei index and found genetic diversity (He) values ranging from 0.082 to 0.323 between populations; the rate was 0.335 at species level. According to Hamrick and Godt (1989), the mean genetic diversity in perennial arboreal species was 0.140, and the population heterozygosity in the species was consistent with "sapucaia" (*Lecythis pisonis),* which recorded 0.32 (Borges, 2015); however, the Shannon index was I = 0.47 in Borges' study. These results suggest that, although the genetic distance value indicated significant differences in the populations, they show high genetic diversity.

The excess of heterozygotes in the population of 10 and 12 loci in the Bottleneck results show recent genetic bottleneck, according to Luikart et al. (1998). The natural or generated wildfires are the main threats for Resex areas, as evidenced by data from our mapping, because fire leads to the loss of individuals and alleles in the population. Deshpande et al. (2001) assure that the explanation for the large number of loci presenting over heterozygosity lies on ISSR markers that tend to generate more polymorphism than other loci. Thus, according to Luikart et al. (1998), recent genetic bottleneck processes, such as that in Resex populations may have been caused by constant wildfires that reduce the effective population.

Theoretically, FST (ϕST) values may range from 0 (no genetic divergence) to 1 (allele fixation); however, the herein observed value was much lower than 1. Wright (1965), for example, suggested the following values in FST interpretation guideline: from 0 to 0.05, little genetic differentiation; from 0.05 to 0.15, moderate genetic differentiation; from 0.15 to 0.25, great genetic differentiation; and values above 0.25 mean excellent genetic differentiation. The ϕST and Fst results (Table 3) indicate that the geographic isolation of bacuri populations in Chapada Limpa Extractive Reserve may be due to constant wildfire events and to loss of individuals in the population, as well as to the species' biology, which has led the population to great genetic differentiation.

The Fst value represents accelerated genetic differentiation process in *P. insignis* populations, and it may lead to genetic isolation in the short-term. Another factor possibly contributing to these results was the use of different bacuri varieties at harvest time. However, some subpopulations may have had their effective population size (Ne, which is defined by the size of an idealized population able to generate the same amount of inbreeding or allele variation in the same frequencies observed in the assessed population) reduced

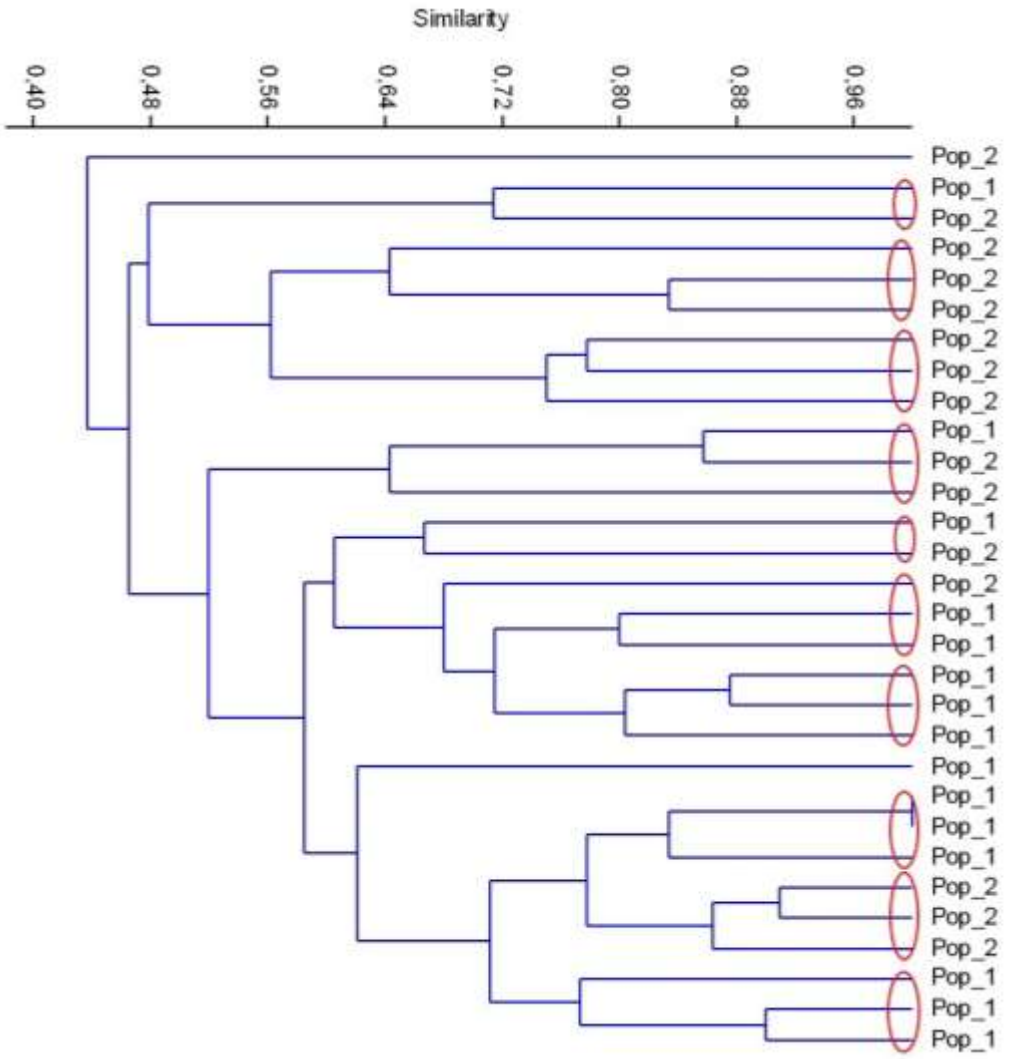

Figure 2. Dendrogram generated in the Past software showing the similarity between individuals belonging to the *Platonia insignis* populations at Chapada Limpa Extractive Reserve, Chapadinha County, Maranhão State.

(Caballero, 1994; Crow and Kimura, 1970; Kimura and Crow, 1963). Therefore, the effective population mainly consists of individuals in reproductive maturation process; hence, the greater the genetic distance, the lower the effective population and the greater the change in the population inbreeding. According to Falconer (1960) and Mettler and Gregg (1973), inbreeding means the mating between kin individuals; however, it depends on the size of the population. Thus, the smaller the population and more isolated it is, the greater the population's possibility to inbreed.

The Shannon index is not based on the population heterozygosity, but on the phenotypic frequency of the amplified fragment (presence or absence of the band) in the population (Yeh et al., 1995; Moura, 2003; Goulart et al., 2005). This index generates values from 0 to 0.73 in a logarithmic scale (Lowe et al., 2004). The present data corroborate those found by Souza (2011), who estimated the Shannon diversity index (H') and found variation from $0.120 \leq H' \leq 0.480$ and classified the population as presenting high diversity indices.

The Jacard's similarity values which are similarity indices for binary data generated a similarity matrix and a dendrogram by comparing individuals in a single population to each other. The dendrogram (Figure 2) revealed 10 small clusters. The cluster analysis is an efficient method to measure bacuri forest clusters, because it illustrates similarities and/or differences between half-sibling progenies and different provenances. Souza (2011) also found genotype sub-clusters wherein half-sibling genotypes were grouped into one subgroup, or into a small number of subgroups. However, Sanches et al. (2015) found Jacard's genetic similarity values between 0.33 and 0.93 in Pará State

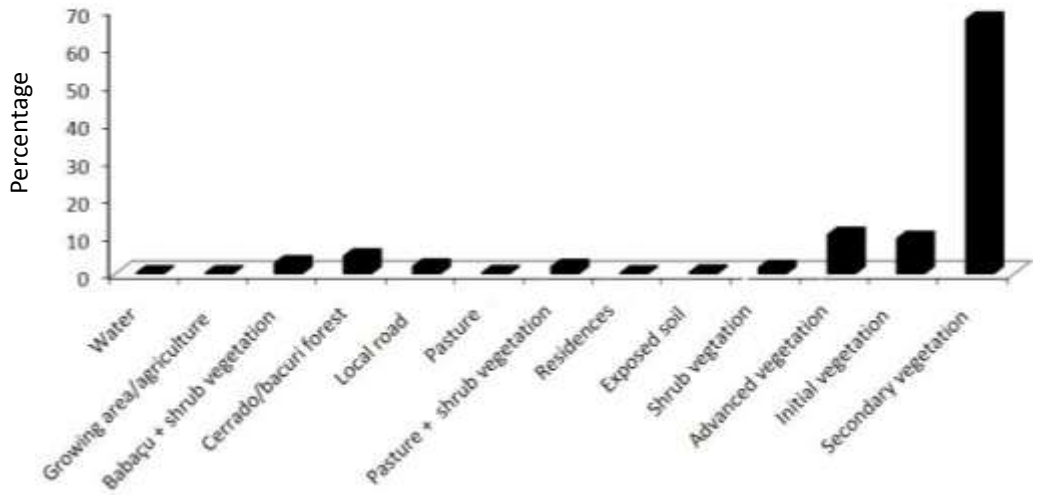

Figure 3. Land use and occupation percentage in Chapada Limpa Extractive Reserve – Chapadinha County, Maranhão State.

progenies. The study by Almeida et al. (2007) stands out among the few on the RAPD molecular marker of bacuri populations in Northern and Northeastern Brazil selecting germplasm genotypes from populations in Pará, Maranhão and Ceará states. These authors evidenced that Maranhão State has the highest degree of polymorphism among other states (93.5%) and indicated the high genetic diversity degree of the species.

Results evidenced that bacuri gradual genetic differentiation at Chapada Limpa Extractive Reserve, even at high genetic diversity may have suffered direct consequences from the intense wildfire events and/or from primary vegetation reduction. The loss of individuals in the population may lead to the loss of heterozygous alleles and to population inbreeding processes that may create genetic bottlenecks. Although Cerrado is a fire-resistant biome, the constant wildfire events of unknown cause may affect soil quality, genetic variability, as well as bacuri production and reproduction in the forest.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Evaluation of serum sialic acid and other risk factors in diabetes mellitus

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The main cause of diabetes mellitus type 2 (DM2) is disturbed carbohydrate metabolism indicated by hyperglycemia. Insulin secretion is disturbed by inflammatory process in the body due to imbalanced diet, hormonal disturbance and genetics. The serum sialic acid has been investigated as a parameter to evaluate intensity of DM2. 200 individuals of both genders were included and were divided into four groups (50 each) which include; Group 1, the control; Group 2, diabetics without complications; Group 3, diabetics with renal complications and Group 4, diabetics with renal complication and retinopathy. All the blood components were estimated by using enzymatic kits. Body mass index (BMI) was also estimated. It was observed that serum sialic acid (mg%) showed significantly higher (P< 0.01) values in diabetics than control and all other risk factors. All other parameters revealed significantly higher differences than control group with diabetic complications. The present findings indicated that elevated serum sialic acid levels predict renal function disturbance. The serum sialic acid may be taken as a strong predictor of renal dysfunction in diabetes.

Key words: Sialic acid, triglycerides, cholesterol, nephropathy, retinopathy, body mass index (BMI).

INTRODUCTION

Diabetes mellitus type 2 (DM 2) is diagnosed by hyperglycemia and is a result of disturbed carbohydrate metabolism due to abnormal insulin secretion and is also associated with several micro vascular complications. DM is controlled by the hormone insulin from beta cells of Langerhans which secrete insulin according to metabolic needs but deviation from normal insulin secretion due to any reason results in raised serum glucose level and leakage in urine when serum glucose exceeds 180 mg% (Guyton and Hall, 2016). During the last few decades,

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DM2 has become very common in human beings around the world due to dietary errors or physiological deviations. Only in USA more than 16 million Americans have DM type 2 (Mehta et al., 2009). In India, diabetics were 40.6 million in 2006 which is expected to go up to 79.4 million by the year 2030 (Pradhan et al., 2001; Ghosh et al., 2016). In addition to this, a great percentage is not aware if they are diabetic. It is proposed that urine and blood sugar should be tested at least once in 6 months so that the disease may be handled in its primary stages (Ghosh et al., 2016).

The diabetic complications may result as cardiovascular diseases, kidney nephropathy, retinopathy or other debilitating diseases (Pickup et al., 1997). There are several metabolites in the body including elevated serum sialic acid which may indicate the presence of DM 2 including raised lipid profile, uric acid or delayed wound healing and are also related with cardiovascular diseases. In this study we have focused serum sialic acid (SA) comparison with other diabetic indicators because sialic acid has been reported as a risk factor depicting acute inflammatory changes in the cell membrane and its damage leading to ischemia in blood vessels, kidney, eyes and brain (Yarema, 2006). It is formed by glucosamine in association with acetyl-L-carnitine (acetyl CA). SA is represented with the formula $C_{11}H_{19}NO_9$. It is also known as N-acetyl neuraminic acid, the predominant sialic acid found in mammalian cells with a molecular weight of 309.2699 gM^{-1} (1 mM = 2.709 mg%) (Chen et al., 1996). The kidney nephropathy with raised Hemoglobin A1c (HbA1c) and other complications such as retinopathy are also common complications of DM 2. Obesity is also a complication of diabetes or vice versa. Among all parameters which indicate status of DM 2, the raised serum sialic acid is an acute reactant of inflammation resulting from elevated blood sugar levels (Ghosh et al., 2016; Cohen et al., 1981). The increased insulin resistance and lower serum sialic acid contents indicate their association with neuramidase biosynthetic enzyme in DM 2 which is indicative of reduced responsiveness to insulin (Salhanick and Amatruda, 1988).

The study was undertaken to determine different parameters such as serum glucose, creatinine, urea, triglycerides, and cholesterol, compared with sialic acid because it is an acute phase indicator of inflammation as a risk factor for diabetes. BMI is also considered because being overweight or obese is mostly associated with diabetes due to disturbed carbohydrate metabolism.

METHODS

In this study a total of 200 individuals of both genders were included which were divided into four groups including control (with no diabetes), diabetics (without complication), diabetics (with nephropathy complications) and diabetics with retinopathy. Each group comprising of 50 individuals subjected to estimation of serum sialic acid (SA), blood sugar, creatinine, urea, hemoglobin A1c (HbA1c), triglycerides and cholesterol as milligram per deciliter with body mass index (BMI). Blood (5 ml) was collected from anti cubital veins in sterile syringes after 12 h fasting from all individuals of each group. The blood for HbA1c was separated in vial having ethylenediaminetetraacetic acid (EDTA). The samples were collected from different hospitals and educated families after getting written consent from each person and results were kept confidential with fictitious numbers. The basal metabolic index was also calculated. The individuals were mixed male, females from 25 to 55 years having diabetes for the last 10 to 12 years and were under medication of one or the other type (tablet or insulin). The patients with cardiovascular diseases, cancer or kidney failure were excluded from the study. The serum was extracted by centrifugation at 2000 rpm and was stored at 4°C in the refrigerator until analyzed (Svennerholm, 1957). HbA1c, an indicator of degree of metabolic control of blood sugar was estimated using the spectrophotometry technique (Crook et al., 2001). The HbA1c kit was supplied by Seimens Company (Hoelzel et al., 2004). The BMI was calculated using formula weight in kg/height in m^2 (Katia et al., 2015). The other risk factor parameters in the serum were also estimated by colorimetric method with spectrophotometer using enzymatic kits from Roche. The data thus obtained was analyzed by SPSS version 18 to find out mean and standard deviation (mean± SD). The BMI is represented as a range for each parameter in each group considering 24.9 as the normal BMI. No complicated case of retinopathy was included in the study and the diabetics with complaint of occasional blurred vision were taken as retinopathy group relying on their eye testing report and no gross retinal symptom was reported. All the data is presented in Table 1 and Figure 1. The study was conducted between May, 2011 and May, 2014 (sample collection took more time). The blood samples were analyzed at Biochemistry Laboratory, Institute of Molecular Biology, The University of Lahore, Andrology Laboratory, Fatima Memorial Hospital, Lahore, Ali Diagnostic Laboratory Ali Hospital Lahore and Biotechnology Laboratory GC University Faisalabad, Pakistan.

RESULTS

The serum sialic acid concentration in non-diabetics (Control) and diabetics with or without complications are given in Table 1 and Figure 1. In order to observe preference of sialic acid over the other risk factors such as serum sugar, serum creatinine, blood urea, serum triglycerides, serum total cholesterol, HbA1c and BMI; the mean ± standard deviation (SD) were compared with SA based on their serum levels in diabetes with control and between diabetic groups for their significance. In no subject was reported urine albumin, therefore, this parameter was not included. The control subjects showed 41.01 ± 9.7 mg% serum sialic acid as compared to 57.25 ± 3.2 mg% in diabetics with no complication, 91.24 ± 1.2 mg% in diabetics with nephropathy and 79.1 ± 1.7 mg% in diabetics with retinopathy and were compared statistically using mean ± SD. The BMI of controls was also compared with other three groups. 50 individuals irrespective of genders were selected in each group in this cross-sectional study. In all groups, the serum SA showed higher levels than control group which had values close to normal physiological levels. The blood sugar also showed significantly higher levels in all the three diabetic groups as compared to the control group. The diabetics with nephropathy showed significantly

Table 1. Serum sialic acid comparison with other kidney dysfunction parameters in diabetes type 2 patients (sampled after 12 h fasting).

Parameter/diabetes Marker	Control (non- diabetic) (Mean± SD; N=50)	Diabetic without complications (Mean ±SD; N=50)	Diabetics with kidney malfunction (Mean ±SD; N=50)	Diabetics with retinopathy (Mean ±SD; N=50)
Serum sialic acid (mg%)	41.01±9.7	57.25±3.2**	91.24±1.2**	79.1.±1.7**
Blood sugar (mg%)	91.25±6.2	188.54±2.3*	261.20±6.2**	254.20±5.1*
Serum creatinine (mg%)	1.32±0.1	1.50±0.1**	1.65±0.6**	1.53±0.3**
Blood urea (mg%)	26.24±2.7	70.23±6.1**	140.60±4.1**	130.1.2±2.1**
HbA1c	6.21±3.4	10.31±1.2**	14.61±2.2**	12.1±3.1**
Serum triglycerides (mg%)	134.14±30.1	200.12±40.3**	270.32±16.5**	262.11±38.2**
Serum total cholesterol (mg%)	189.37±37.2	242.40±42.3**	285.12±36.2**	261.43±43.7**
BMI (Kg/ m^2)	23.34to24.01[A]	32.51to39.10[A]	34.21to40.01[A]	33.13to38.71[A]

**Significant at P<0.01; *Significant at P<0.05; n = 50 individuals in each of four groups. [A]BMI range in each group.

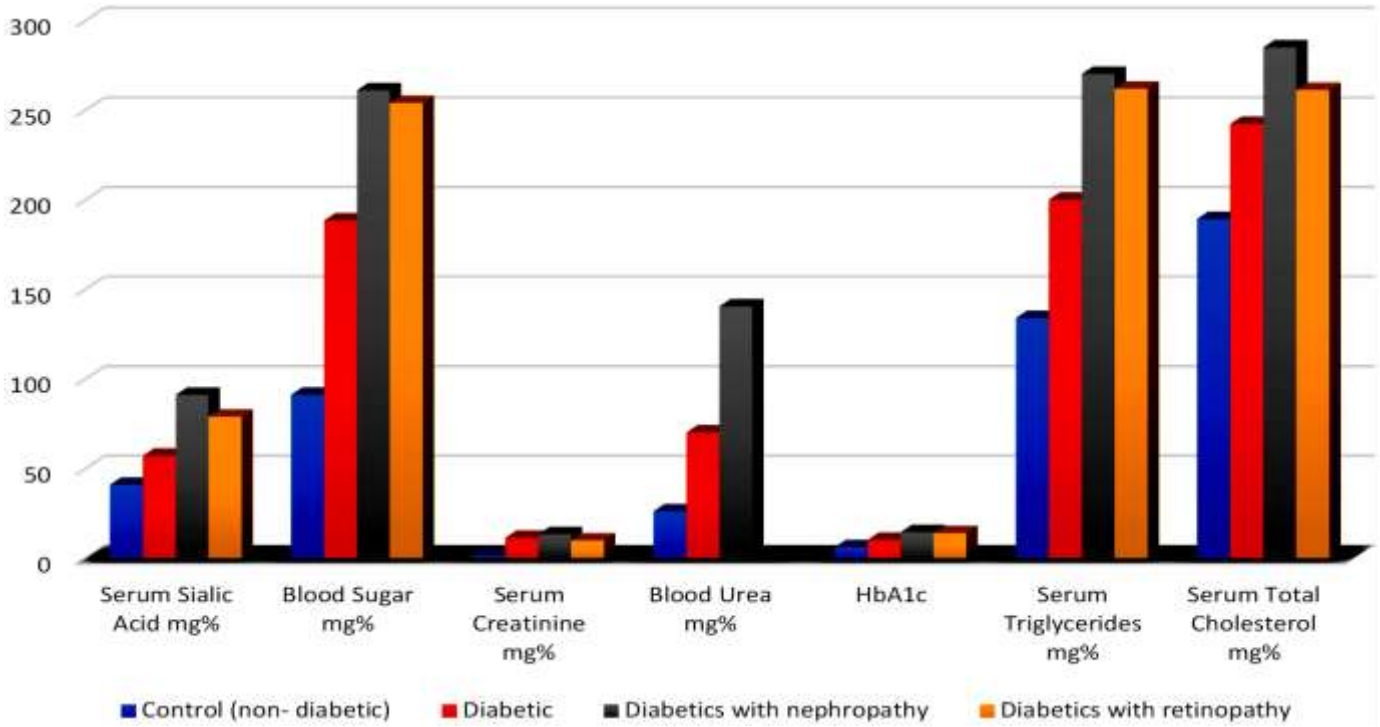

Figure 1. Serum sialic acid comparison with other nephropathy parameters in diabetes type 2 patients (After 12 h fasting) (n=50).

higher (P<0.01) blood sugar levels, that is, 261.20 ± 6.2 and 254.20 ± 5.1 mg% in diabetics with retinopathy as compared to 188.54 ± 2.3 mg% in diabetics without complications. All subjects, except the control group, showed significantly higher blood sugar levels than 91.25 ± 6.2 mg% blood sugar.

The serum creatinine also revealed significant differences as compared with the control which was 1.32±0.1 mg%, 1.5±0.1 mg% in diabetics without complications, 1.65±0.6 mg% in diabetics with complications and 1.53±0.3 mg% in diabetics with retinopathy, respectively. However, the diabetic groups did not show significant differences within diabetic groups but had significantly higher values than the control group.

The blood urea was 26.24±2.7 mg% in the control group, 70.23±6.1 mg% in diabetics without complications, 140.60±4.1 mg% in diabetics with complications and 130.12± 2.1 mg% in diabetics with retinopathy.

The values for HbA1c also showed differences which had 6.21± 3.4 for control group, for diabetics without complications was 10.31±2.12, for diabetics with complications was 14.61± 2.2 and for diabetics with retinopathy was 12 .1± 3.1.

Serum triglycerides revealed significant differences as 134.14±30.1 for control group, 200.12±40.3 mg% for diabetics without complications, 270.32±16.5 mg% for diabetics with complications and 262.11± 38.2 mg% for diabetics with retinopathy.

The results for serum total cholesterol revealed major differences in the values that were, 189.37± 37.24 for control group, 242.40±42.3 mg% for diabetics without complications, 285.12± 36.2 mg% for diabetics with nephropathy and 261.43± 43.7 mg% for diabetics with retinopathy. The differences in serum values can be visualized at a glance in Figure 1.

The values for BMI were estimated as range for each group which showed differences approaching over weight and obesity levels that were, 23.34 to 24.01 m^2 for control group, 32.51 to 39.10 m^2 for diabetics without complications, 34.21 to 40.01 m^2 for diabetics with nephropathy and 33.13 to 38.71 m^2 for diabetics with retinopathy.

DISCUSSION

Finding diabetic individuals for the study was a bit hard task but those who understood the research motive, gave consent without argument and many wanted results which were provided confidentially to desirous individuals. Most of the patients were only relying on blood and urine sugar tests with long intervals and continued treatment prescribed years back by a physician. Therefore the present results of their tests gave them a good guideline for treatment. The results presented in Table 1 and Figure 1 pertaining to different diabetic conditions may be helpful for further treatment of diabetes. The elevated serum sialic acid levels as compared to control subjects were indicative of diabetic complications. The serum SA within diabetic groups also showed significant differences. The results are in agreement with the findings of Shahid and Mehboob (2006) who conducted a similar study (Mahboob, 2008). These findings also correlate with the results presented by Crook (1993). The present findings in Table 1 and Figure 1 showed that serum SA can be trusted as indicator of diabetes.

The blood sugar level of diabetics with or without complications was significantly higher as compared to control group indicating the effect of diabetes on kidney function due to elevated levels of different parameters used for diabetes diagnosis. The findings are similar to the results presented by Linderberg et al. (1991). Almost parallel findings were reported for blood glucose level by Gavella et al. (2003) in different diabetic complications. These results indicate that diabetes has different effects on kidney function as the level of blood sugar increases therefore dietary control along with medication is equally important.

The kidney function parameters such as serum creatinine and urea in diabetics were also higher in all diabetic groups as compared to control group. However, the serum creatinine in diabetics without complications, diabetics with complications and diabetics with retinopathy did not show significant differences yet the creatinine level had entered critical threshold than can be accommodated by nephrons. Similarly urea was a few times higher in diabetics as compared to control group which indicates the protein metabolism disturbances and needs specific management.

HbA1c is an indicator of kidney ability to handle glucose and the damage done to kidney was compared in all three diabetic groups. The HbA1c level was significantly higher than control group showing the damage so far done to kidney cells for handling serum glucose. These findings match with the results reported by Nayak and Bhakhta (2005) who conducted a similar study. Similar results were reported by Svennerholm (1957) indicating diabetic damage on kidney.

Similarly the serum triglycerides and total cholesterol concentration was higher in diabetics as compared to control group. The triglycerides and cholesterol also indicate cardiovascular diseases along with diabetes. At the same time higher levels of TG and cholesterol may also be hazardous for kidney function and indicate several other complications in addition to diabetes. These findings are similar to the findings of Jeremy (2003) and WHO (1985). The triglycerides and cholesterol are alarming indicators for hypertension and cardiovascular diseases in addition to adverse effect on kidney function. Similar findings on TG and cholesterol were reported by Pickup et al. (1997). This shows that elevated serum sugar is closely associated with other metabolites creating a risk for cardiovascular and renal normal functioning.

The basal metabolic index (BMI) is closely related with body mass, body fat and body weight (Table 1). It is evident that BMI in all the three diabetic groups was higher than control group but between diabetic groups, the BMI was not grossly different. The results in Table 1 show that BMI ranges of diabetic groups were even higher than the overweight level which is very alarming signal for several diseases including hypertension, diabetes and cardiovascular diseases.

Conclusion

From the results presented in Table 1 and Figure 1, that serum SA is very closely related with different diabetic conditions including nephropathy or retinopathy. However the glycemic control indicated by blood sugar and HbA1c and the renal functioning threshold estimated by serum urea, creatinine contents along with body mass index all go together in damaging kidneys if not controlled. It is suggested that all the parameters discussed are of similar importance for diabetic control and treatment. It may be inferred that serum SA is more closely related with diabetic complications than other parameters which show acute stage and also has relationship with cardio-vascular diseases. Therefore, elevated level of serum SA may be taken as an important indicator of diabetic nephropathy.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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