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Influence of AM fungi and its associated bacteria on growth promotion and nutrient acquisition in grafted sapota seedling production

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Abstract: A study was undertaken to know the effect of co-inoculation of Arbuscular Mycorrhizal (AM) fungi and its associated bacteria on enhancing AM root colonization, growth promotion and nutrient acquisition in grafted sapota plants. The best mycorrhiza associated bacteria i.e. *Pseudomonas putida* (HM590707) isolated from *Funneliformismosseae* spore was evaluated along with AM fungi for growth promotion and AM fungal colonization in grafted sapota plants.The combined application of *P.putida*along with AM fungi significantly increased plant height (39.67 %), stem girth (3.2 cm), total biomass (66.8 g plant^{-1}), AM root colonization (73.4 %)and plant nutrient concentrations *viz.,* N (2.52 %), P (0.18 %), K (2.90 %), Fe (428.4 ppm) and Zn (21.40 ppm) as compared to uninoculated control. This finding clearly demonstrated that grafted sapota plants can be successfully established by combined inoculation of AM fungi and its associated bacteria which have a greater impact on healthy grafted plants.

Keywords: Arbuscular Mycorrhizal fungi, Grafted sapota plants, Mycorrhiza associated bacteria

INTRODUCTION

Positive plant-microbe interactions are considered as the most potent and primary indicators of plant health, soil fertility and for sustainable crop production systems (De Souza *et al.,* 2015). The plant growth promoting bacteria (PGPB) which is closely associated in rhizosphere or on the root surface or as endophytes are found to be more beneficial for growth promotion and protection of plants from biotic and abiotic stresses (Dimkpa *et al.,* 2009; Glick, 2012). One of the most promising microorganisms which colonize the plant root system efficiently and enhance plant growth promotion are arbuscular mycorrhizal (AM) fungi. AM fungi forms a symbiotic association which benefits plant with increased uptake of nutrients and water (from soil interphase and inturn utilizes carbon provided by the plant for its growth and development (Shamshiri *et al*., 2012).

AM fungal spores are known to provide shelter for beneficial bacteria which produce stimulatory compounds such as flavonoids, sugars and volatile compounds (Hildebrandt *et al*., 2006; Xie, 1995; Lagrange *et al*. 2001) for better AM colonization, spore germination and extra radical hyphal growth (Nazir *et al.*2010).These bacteria are known as mycorrhiza helper or associated bacteria (Garbaye, 1994) and are fungi specific or host specific (Pivato *et al.* 2008; Zhang *et al.,* 2016) (Pivato *et al.,* 2008). AM fungal interactions with bacterial communities directly influence plant growth in several ways as they alter nutrient supply, provide phosphorus to the host plant (Barea *et al.,* 1997, Bonfante and Anca, 2009). These mycorrhiza associated bacteria (MAB) are known to secrete metabolites, cell wall degrading enzymes, change soil pH in support of AM colonization and promote spore germination (Bharadwaj *et al.,* 2011). Besides, these MAB act as effective plant growth promoters, nutrient solubilizers and biocontrol agents in some of the fruit crop seedlings (Panneerselvam *et al.,* 2012, Sukhada *et al.,* 2013) like guava.

Sapota (*Manilkara achras* (Mill.) Forsberg) is a tropical fruit crop mainly cultivated for its delicious fruits. Sapota provides a continuous crop with a wide range of adaptability to different agro-climatic conditions, hence, the area and production under this crop is increasing in a large extent. Sapota is being propagated both by seed and vegetative methods, but most commonly vegetative propagation was used due to slow growing nature of seedlings. The vegetative methods involve grafted seedlings, rootstock grafting and air-layering.

Several reports suggested that application of microbial inoculants is essential to improve the plant growth (Kloepper *et al.,* 1980, Panneerselvam *et al.,* 2012). Similarly, reports on use of AM fungi and its associated bacteria in growth promotion of grafted sapota plants were not yet recorded. In this study, an attempt

was made for the first time to study the effect of AM fungi and its associated bacteria on stimulation of mycorrhizal colonization, growth promotion and in nutrient acquisition for sustainable grafted sapotaseedling production.

MATERIALS AND METHODS

Isolation and Identification of mycorrhiza associated bacteria: The rhizosphere soil samples were collected from different sapota fields in Karnataka, India and the AM fungal spores from these samples were isolated by adopting wet sieving and decanting technique (Gerdemann and Nicolson, 1963). The isolated spores were identified based on spore morphology (Schenck and Perez, 1990) by using Stereozoom microscope (Olympus SZX9-Japan). The surface sterilized AM fungal spores (*F.mosseae*) from sapota rhizosphere treated with 5 % Chloramine-T (BDH Inc., Toronto, Canada) for 30 min (Walley and Germida, 1996) were crushed with sterile water and then plated out in nutrient agar mediumand incubated at 30 ^{o}C for 48 hours. The bacterial isolates thus obtained were screened for their plant growth promoting attributes such as phytohormone production (IAA and GA_3), siderophore production, nutrient solubilization and mycorrhizal colonization (Panneerselvam *et al*., 2013). Based on the above parameters, four mycorrhiza associated bacteria (MAB) were selected andidentified by using16S rRNA technique.Among the four MAB isolates, *Pseudomonasputida* (Genbank accessions HM590707) was selected based on its potential in enhancement of mycorrhizal colonization and growth promotion in sapota seedlings (Panneerselvam*et al*., 2013), for the present study.

Plant growth response to AM fungal inoculation and its associated bacteria: Five month old grafted sapota (variety: Cricket Ball) plants raised by using khirni as standard root stock received from Nursery Unit, ICAR-IIHR, Bengaluru were used in this experiment. The experiment comprised of four treatments with five replications arranged in completely randomized design. The treatments include an uninoculated control, *P.putida* alone, *P.putida* plus AM fungi and AM fungi alone.The mixed AM fungal inoculum (*F.mosseae, R.fasciculatus* and *R.intraradices*) was used. In this experiment, 20 g of AM fungal inoculum (70 -80 spores g^{-1} substrate) and 10 g lignite carrier based *P.putida* (10^8 cells g^{-1}carrier) were applied per seedling. The seedlings were allowed to grow and no pesticide or fertilizer was applied. The grafted plants were watered once in a day during the period of experiment.

After 18 months of inoculation, plants from each treatment were randomly harvested including complete root systems and observations on growth parameters, plant height (cm), stem girth (cm) and dry biomass (g plant^{-1}) of root and shoot were recorded. Dry biomass of shoot and root were determined after drying the tissue to a constant weight in an oven at 80 ºC for 48–72 hours. The AM fungal association in each specimen was examined in the roots following the staining method of Phillips and Hayman (1970), and then calculated as a percentage of mycorrhizal colonization by using theformula as given below:

Per cent AM fungal Colonization (%) = Number of infected root segments /Total number of root segments examined x 100

AM fungal spore content of soils was assessed using the wet sieving and decanting method (Gerdemann and Nicolson, 1963). Estimation of plant nutrient uptake *viz.,* N, P, K, Ca, Mg, Mn, Fe, Zn and Cu in grafted sapota plants was analysed by following the standard procedures (Humphries, 1956, Jackson, 1973).

Statistical analysis: The data were analysed using SAS GLM V 9.2 (Statistical Analysis System, 2008).

Table 1. Effect of AM fungi and *P.putida*on growth promotion in grafted sapota plants (After 18 months of inoculation).

Treatments	Shoot height (cm)	Stem girth(cm)	Fresh weight (g plant^{-1})		Dry weight (g plant^{-1})		Total biomass (g plant^{-1})
			Shoot wt.	Root wt.	Shoot wt.	Root wt.	
T1- Control	31.83	2.41	81.9	33.9	32.7	17.4	50.1
T2- AM fungi alone	39.46	2.80	92.6	47.4	44.9	18.8	63.7
T3- AM fungi+*P.putida*	39.67	3.20	97.1	49.2	45.8	21.0	66.8
T4- *P.putida* alone	39.25	2.61	85.8	44.3	35.4	18.7	54.1
SEM	0.20	0.02	0.48	0.27	0.26	0.10	0.36
CD (p=0.05)	0.43	0.04	1.05	0.60	0.57	0.23	0.79

Values are mean of five replications, SEM-Standard error means, CD (p=0.05)-Critical difference at 5 % level

Table 2. Effect of AM fungi and *P.putida*on plant nutrient uptake in grafted sapota plants (After 18 months of inoculation).

Treatments	N (%)	P (%)	K (%)	Ca (%)	Mg (%)	Mn (ppm)	Fe (ppm)	Zn (ppm)	Cu (ppm)
T1- Control	2.10	0.11	2.18	2.01	0.48	2.20	390.7	14.10	10.90
T2- AM fungi alone	2.31	0.16	2.56	3.35	0.78	7.30	412.4	19.40	9.50
T3- AM fungi+*P.putida*	2.52	0.18	2.90	1.82	0.42	6.80	428.4	21.40	6.70
T4- *P.putida* alone	2.29	0.13	2.34	2.65	0.56	1.00	398.1	18.20	5.80
SEM	0.01	0.00	0.02	0.02	0.00	0.08	2.04	0.12	0.06
CD(p=0.05)	0.03	0.00	0.03	0.04	0.01	0.17	4.45	0.26	0.14

SEM-Standard error means, CD (p=0.05)-Critical difference at 5 % level

Data were subjected to one-way analysis of variance. Percentage of AM colonization and spore numbers were arcsine and square-root transformed, respectively, to ensure homogeneity of variance before analysis (Gomez and Gomez 1984). Treatment differences were evaluated using least significant difference at $p< 0.05$.

RESULTS AND DISCUSSION

Plant growth response to AM fungal inoculation and its associated bacteria: The growth parameters such as plant height, stem girth, plant dry weight were increased due to coinoculation of AM fungi with *P.putida* when compared to individual inoculation and uninoculated control (Table 1). Application of AM fungi with *P.putida* (T3) significantly increased shoot length, stem girth and total dry biomass by 24.63, 32.78 and 33.33 per cent, respectively, as compared to control. Among the treatments, there was no much variation in plant height, but significant variation was observed in stem girth and plant dry biomass.The recent findings indicated that some bacteria, closely associated with the surface of AM fungal spores (Selvakumar *et al.,* 2016) and play an important role in enhancing mycorrhizal colonization with host plants. Bharadwaj (2007) reported that MAB isolates of *Pseudomonas* sp. and *Stenotrophomonas* sp. isolated from *G.mosseae* and *G.intraradices* stimulated AM colonization in potato roots under greenhouse conditions. In another study, *Paenibacillus* isolate stimulated the growth of *G.intraradices* in the formation of newly colonizing spores (Hildebrandt *et al.,* 2006).The information available from other fruit crops indicated that the mycorrhized seedlings in rootstock stimulated plant growth than the non [mycorrhized seedlings. The combined application of AM fungi and rhizobacterial strains found to increase the plant growth in citrus rootstocks (Chiquito-contreras *et al.,* 2012). The AM fungal consortium (*Sclerocystisdussii*, *G.fasciculatum, G.intraradices* and *G.monosporum)* treated Jamun (*Syzygiumcuminii*Skeels) seedlings recorded higher plant growth (Devachandra *et al.* 2008a) than non-mycorrhizal grafts. In mango rootstocks, there was 41.34 per cent higher biomass in *G.margarita* plus *G.fasciculatum* applied treatment as compared to uninoculated control (Patil and Patil, 2007).

Plant nutrient uptake in grafted sapota plants: The plant nutrients *viz*., nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and micronutrients*viz.,* Manganese (Mn), Iron (Fe), Zinc (Zn) and Copper (Cu) were analysed after 18 months of inoculation and the results are given in Table 2. Grafted sapota plants inoculated with AM fungi and *P. putida* either individually or in combination enhanced total nitrogen concentration over uninoculated control after 18 months of sampling. The combined application of AM fungi and *P.putida* recorded significantly higher nitrogen (2.52 %), phosphorus (0.18 %) and potassium concentration (2.9 %) as compared to control.

Plant samples collected either from *P.putida* or *P.putida* with AM fungi inoculated treatments recorded significantly higher micronutrient concentrations like Fe, Zn and Mn as compared to uninoculated control (Table 2). Most commonly, the grafted sapota plants inoculated with the combination of cultures (T3) showed significantly higher concentrations of micronutrients Fe (428.4 ppm), Zn (21.4 ppm) and Mn (6.8 ppm) when compared to uninoculated control.

Jacobsen *et al.* (1992) and Jeffries (1987) reported that AM fungi played an important role in increasing the uptake of slow diffusing ions and improved productivity in low fertility soils. The acquisition of orthophosphate and other mineral nutrients such as Zn was improved in the mycorrhizal plants (Thonar *et al*., 2011) when compared to non-mycorrhizal plants. Enhanced acquisition of P, Zn, Cu and Fe by mycorrhizal plants has been reported by Abbaspour *et al.* (2012). In Pistachio seedlings, the nutrient *viz.,* K, P, N, Ca, Fe, Cu and Zn concentrations were higher in mycorrhizal seedlings than uninoculated control (Abbaspour, 2016). Similarly, increased uptake of P and N nutrients was reported in soyabean and cotton crops inoculated with AM spores of *Rhizaophagusclarus* (Cely *et al.,* 2016).

Effect of AM fungi and *P.putida* on AM colonization and spore number in grafted sapota plants: The AM fungal colonization and spore number observed from different treatments are given in Table 3.Among the treatments, application of AM fungi with *P.putida* recorded significantly higher root colonization (73.4 per cent) and spore number (17.2 spores g^{-1} soil) followed by individual inoculation of AM fungi alone (70.8 per cent and 15.2 spores g^{-1} soil) and *P.putida* alone (69.5 per cent and 12.1 spores g^{-1} soil).

Table 3. Effect of AM fungi and *P.putida* on AM colonization and spore number in grafted sapota plants (After 18 months of inoculation).

Treatments	AM Colonization (%)	AM Spore Number (spore g^{-1} soil)
T1- Control	63.2	11.5
T2- AM fungi alone	70.8	15.2
T3- AM fungi+*P.putida*	73.4	17.2
T4- *P.putida* alone	69.5	12.1
SEM	0.35	0.10
CD(p=0.05)	0.77	0.22

SEM-Standard error means, CD (p=0.05)-Critical difference at 5 % level

The lowest AM colonization (63.2 per cent) and spore number (11.5 spores g^{-1} soil) was recorded in uninoculated control.

Mycorrhiza-associated bacterial (MAB) communities have been investigated by many researchers. This association helps in plant growth promotion not only by improving mycorrhizal root colonization and stimulating extra radical hyphal growth but also by facilitating AM fungal spore germination (Gryndler *et al.,* 2000). Also, the inoculation of AM fungi and MAB enhanced uptake of essential nutrient, such as phosphorus, nitrogen and zinc in crop plants (Artursson *et al*., 2006; Salimpour *et al*., 2010). *Paenibacillusvalidus* supported the growth and sporulation of *G.intraradices* (de Boer *et al*., 2005) and this association is highly efficient in sustaining fungal growth and germination of new spores by release of sugars (Hildebrandt *et al*., 2006) and some unidentified compounds in the rhizosphere of plant system (Xie, 1995; Lagrange *et al*., 2001). This finding indicated that AM fungus can grow independently on the host plant in the presence of their closely associated bacteria (Hildebrandt *et al.,* 2006). Panneerselvam *et al*. (2012) proved that combined inoculation of *G.mosseae* and its associated bacteria (*Pseudomonas* sp.) in guava seedlings significantly increased the AM colonization (86.2 per cent) as compared to individual inoculantion of *G.mosseae*.

Conclusion

This study revealed that the coinoculation of AM fungi with *P.putida* is essential for enhancing plant growth, AM colonization and nutrient acquisition in grafted sapota plants. These findings will have a large impact on establishment of grafted sapota seedlings production in the near future. First time in India, it was proved that application of AM fungal spore associated bacteria along with AM fungi is essential for enhancing mycorrhizal colonization, growth promotion and plant nutrient uptake in perennial fruit crops particularly for sapota grafted seedlings production.

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