Applied Biological Research 14 (2): 168-175, 2012

IMPACT OF BIOFERTILIZERS ON GROWTH, NUTRIENT UPTAKE, YIELD, METABOLISM AND RHIZOSPHERE ENZYME ACTIVITIES OF POMEGRANATE (*Punica granatum* L.) cv. 'Kandhari Kabuli'

Muzaffar Mir[*], G.I. Hassan, K. Sheikh[#] and Sanjeev Kumar Sharma[§]

Department of Fruit Science, [§]Department of Microbiology, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh - 173 230 (India)
*[#]Department of Entomology, CS K Himachal Pradesh Krishi Vishwavidyalaya, Palampur, Himachal Pradesh - 176 062 (India); **email: *rajamuzaffar16@yahoo.com*

(Received 23 May, 2012; accepted 15 September, 2012)

ABSTRACT

Nursery and field experiments were carried out to assess the effectiveness of N_2-fixing bacteria, phosphate solubilizing bacteria and arbuscular mycorrhizal (AM) fungi, alone or in combination, on the growth and biomass production of pomegranate (*Punica granatum*). The study revealed significant improvement in nursery seedlings growth by biofertilizers use. Combined treatment of *Azotobacter chroococcum* + *Glomus mosseae* proved most effective in enhancing the rhizosphere microbial activity and concentration of various metabolites and nutrients in pomegranate in nursery experiments as well as helped in better plant establishment under field conditions. A significant improvement in plant height and fruit yield was observed in pomegranate plants under field conditions. Biofertilizer application also improved plant nutrient uptake and rhizosphere microbial activities. Results indicate that biofertilizer technology may be adopted for the establishment and development of horticultural plant species in rainfed agro-ecosystem.

Keywords: *Azotobacter*, mycorrhizal fungi, pomegranate, soil enzymes, PSB, yield

INTRODUCTION

Pomegranate (*Punica granatum* L.) is an important commercial fruit plant belonging to family Punicaceae. The plant is drought tolerant and winter hardy, and also thrives under rainfed conditions. Pomegranate is a good source of protein, carbohydrate, minerals, antioxidants and vitamins (A, B and C). It has been used in controlling diarrhea, hyperacidity, tuberculosis, leprosy, abdominal pain and fever. Pomegranate juice contains antioxidants such as soluble polyphenols, tannins, anthocyanins and may have antatherosclerotic properties (Michel *et al.*, 2005). Also, it can be used in the treatment of cancer and chronic inflammations (Ephraim and Robert, 2007). In India commercial pomegranate orchards are found in rained areas, which characteristically have nutrient-deficient soils low in organic matter, irregular distribution of rainfall and generally experience water deficiency during plant growth period (Panwar and Tarafdar, 2006). Vegetation of rainfed areas is often hindered by the lack of resident microflora which may act both as source and sink for essential plant nutrients and is of fundamental significance in nutrient transformation. Production of horticultural crops has undergone significant changes in recent years due to the development of innovative technologies including integrated nutrient management practices involving use of biofertilizers, which include phosphate-solubilizing bacteria (PSB), symbiotic and non-symbiotic N_2-fixing bacteria and arbuscular mycorrhizal (AM) fungi. The use of biofertilizers for improving plant growth and yield has gained momentum in

recent years keeping in view the hazardous effects of chemical fertilizers. Reports reveal that nitrogen-fixing bacteria and AM fungi significantly enhance the growth and production of various fruit plants (Khanezadeh *et al.,* 1995) and improve microbial activities in rhizosphere (Kohler *et al.,* 2007). In present investigation an attempt was made to assess the impact of biofertilizers such as *Azotobacter chroococcum*, phosphate-solubilizing bacteria and mycorrhizal fungi on growth, nutrient uptake and rhizosphere microbial activity of pomegranate under nursery and field conditions.

MATERIALS AND METHODS

The nursery and field experiments were conducted on pomegranate cv. 'Kandhari Kabuli' during 2008 to 2011. The experimental farm is located at 30° 50' 45" latitude and 77° 88' 33" longitude at an elevation of 1320 m masl, representing the mid hill zone of Himachal Pradesh (India). The climate of the area is typically sub-temperate with annual rainfall ranging between 800-1300 mm. The orchard soil was sandy in texture with pH 6.55, electrical conductivity 0.59 dS m^{-1} and organic carbon content 0.61%. Water holding capacity, bulk density and porosity of surface soil (15 cm depth) were 31.65, 1.30 and 48.50%, respectively. The initial available soil N, P and K contents were 309.8, 11.7 and 342.8 kg ha^{-1}, respectively. DTPA extractable micro-nutrients viz., zinc, manganese, iron, copper and boron were 1.97, 45.72, 57.24, 2.47 and 0.70 ppm, respectively. Strains of *Azotobacter* (a free living non-symbiotic N_2-fixing bacterium) were isolated from the roots and rhizosphere soil of field-grown pomegranate using nitrogen-free semi-solid malate medium (Day and Dobereiner, 1976) and Jensen's nitrogen-free agar medium (Jensen, 1954), respectively. The strains were got identified through the Department of Microbiology, Indian Agriculture Research Institute (IARI), New Delhi (India). The pure cultures of these strains were maintained in the Department of Microbiology, University of Horticulture and Forestry, Solan, (HP) using above-mentioned media. After 3 days growth, the cells were centrifuged, washed twice in sterile distilled water and suspended in 0.15 M phosphate buffer at pH 7.0. Ten milliliters of cell suspension, having 108 cells mL^{-1}, was used as inoculum for both bacterial types at the time of planting (rainy season) pomegranate cuttings (20 cm long). The spores of AM fungi were extracted from the rhizosphere soils of field grown pomegranate plants by wet sieving and decanting technique (Gerdemann and Nicolson, 1964). The spore number was estimated by the method of Gaur and Adholeya (1994) and spore density expressed as the number of spores 50 g^{-1} soil. The AM spores were identified of upto species level on the basis of size and colour of spores, wall layers and hyphal attachment using the identification manual of Schenck and Perez (1990) and as per the descriptions of International Collection of Vesicular and Arbuscular Mycorrhizal Fungi (*http://invam.wvu.edu)*. The AM fungi were identified as *Glomus fasciculatum* (Gf) and *G. mosseae* (Gm). The pure cultures were maintained on pearl millet/wheat plant roots under sterile condition. Soil including root bits (10 g) containing about 10–12 viable AM fungal propagules g^{-1} was used as inoculum and spread as a thin layer 2 cm below the soil surface in polythene bags (30 × 10 cm) containing 900 g substrate (soil: farm yard manure: pond silt, in the ratio of 3:1:1 w/v). In control no inoculum was added to the polythene bags.

Nursery experiments

Semi-hard wood cuttings of pomegranate (5-month-old cuttings) were kept in 250 ppm indole butyric acid (IBA) solution for 12 h and then one each planted in polybags. The experiment, laid out in a complete randomized design, consisted of 8 treatments [A. *chroococcum* (Ac), *G. mosseae* (Gm), *G. fasciculatum* (Gf), phosphate solubilizing bacteria (PSB), Ac + Gf, Ac + Gm, Ac + PSB and control] with each treatment replicated 20 times. Dual inoculations of Ac + Gm, Ac + Gf and Ac + PSB were selected based on the preliminary data obtained on various combinations of AM fungi, N_2-fixing bacteria and PSB.

After sampling rhizosphere soil alongwith root fragments of 4-month-old seedlings, grown in polythene bags, 10 plants from each treatment were harvested and leaf area (using leaf area meter, C-203, USA) and total chlorophyll (Arnon, 1949) were estimated. Total phenols, reducing sugars and

amino-nitrogen in leaves were analyzed from the alcohol extracts (Mahadhevan *et al.*, 1965). Shoot along with leaves were dried at 65°C till constant weight and dry weight recorded. The percent root colonization, after 4 month's seedling growth, was determined by root slide technique (Read *et al.*, 1976) after clearing 1 cm root segment with KOH and staining with trypan blue. Dehydrogenase activity was assayed by the method of Tabatabi (1982). The soil samples were incubated with 2,3,5-triphenyl tetrazolium chloride, and production of triphenyl formazone determined spectrophotometrically. Hydrolysis of fluorescence diacetate (FDA) was determined by the standard procedure of Schnurer and Rosswall (1982) and the fluorescein released quantified spectrophotometrically. For this, soil sample (0.1 g) was placed in plastic tubes and 10 mL sterile potassium buffer (pH 7.6, 60 mM) added to it. The reaction started after the addition of fluorescein diacetate (1 mg mL^{-1} in acetone). Tubes were sealed and incubated at 37°C for 4 h. After incubation, 10 mL acetone was added to stop the reaction and after centrifugation (3200 rpm) the optical density of supernatant was determined at 490 nm. Total nitrogen-fixing potential (nitrogenase activity) of soil was determined by incubating 50 mg soil in 7 mL test tube containing 3 mL nitrogen-free semi-solid malate medium for 48 h at 30°C. The cotton plugs were replaced with suba-seals and 10% air was replaced with acetylene (C_2H_2) and the ethylene (C_2H_4) produced was estimated using AIMIL-Nucon Gas chromatograph fitted with Porapak-N column (2 × 0.003 m) with N_2 as carrier gas at a flow rate of 25 mL min^{-1}. Nitrogenase (N_2-ase) activity was expressed as n mol C_2H_4 produced h^{-1} (Rao and venkateswarlu, 1982). For alkaline phosphatas activity in rhizosphere soil, the ρ-nitrophenyl phosphate was used as a substrate in a borex-NaOH (pH 9.4) buffer (Tabatabi and Bremner, 1969). Leaf sample (1 g) was used for analyzing total nitrogen (by Microkjeldhal method), phosphorus (by vanado-molybdo-phosphoric yellow colour method), potassium (by flame photometer), calcium and magnesium (by titrimetry employing disodium salt of EDTA) after tri-acid digestion (Jackson, 1967). Copper, zinc, manganese and iron were estimated by using atomic absorption spectrophotometer (AAS 4141).

Field experiments

The nursery seedlings, 30 for each treatment, were transplanted in Research Field of Department of Fruit Science, UHF, Solan during January 2009 in rows with 6.0 m inter- and intra-row spacing. For this purpose, pits of 0.6 x 0.6 m size were dug and filled with 30 kg soil mixture (same as used in plantlets production) and added with 100 g endosulfan dust (4%). The plants were irrigated at fortnight intervals. Immature fruits along with few leaves and young branches were removed during the first season (November-February, 2009). After every winter season pruning was done and weak/dead limbs and basal suckers removed to give proper shape to plants to encourage the growth of new spurs in each season and help the plant to sustain more fruit weight. Pruned materials (weak and dead shoot portion along with leaves) weights were recorded after drying in a hot-air oven to a constant weight and ground to a fine powder. Plant canopy was calculated using the formula πr^2 in which radius mean was measured from all the directions of crown. Plant height, canopy, pruned material and fruit yield were recorded yearly for next 3 years (2009, 2010 and 2011).

RESULTS AND DISCUSSION

Impact on plant growth and metabolites

Sprouting of pomegranate occurred in 6-9 days in biofertilizers inoculated cuttings while it took 8–10 days longer in non-inoculated controls. Also, higher number of branches was observed in inoculated cuttings, 4 months after inoculation, as compared to uninoculated control (Table 1). Early sprouting in inoculated treatments may be due to the action of plant growth regulators mainly indole butyric acid secreted by both nitrogen fixers and AM fungi (Luis *et al.*, 2003). Biofertilizer inoculation enhanced shoot dry weight by 16-36%. The inoculation effect varied among treatments with effect maximum in dual inoculation treatment and minimum in control. However, no significant difference was observed in the number of branches between various biofertilizers used. A significant increase (27.4-57.6%) was observed

in leaf area. The increase was maximum in dual inoculated seedlings i.e. in *A. chroococcum* + *G. mosseae* treatment followed by *A. chroococcum* + *G. fasciculatum* treatment. Increased leaf area with AM fungi treated plants might be due to the growth promoting effect of fungus and *Azotobacter* that improved P and N availability and thereby caused higher protein synthesis and improved morphological growth (Singh and Singh, 2004). Similar observations of enhanced leaf area was made in different fruit plants upon inoculation with AM fungi (Khanezadeh *et al*., 1995), phosphorus solubilizing bacteria (Karlidaga *et al*., 2007) and *Azotobacter* + *G. fasciculatum* (Sharma *et al*., 2008; 2009).

Biofertilizer inoculated plants showed significantly higher total chlorophyll content and more accumulation of reducing sugars, total phenols and amino-nitrogen in 4 month old inoculated plants than uninoculated ones (Table 1). Total chlorophyll was high in seedlings dually inoculated with *A. chroococcum* + *G. mosseae* followed by *A. chroococcum* + *G. fasciculatum*. A similar trend was found in total phenols, amino nitrogen and reducing sugars contents. High total chlorophyll content and more accumulation of various metabolites (reducing sugar, total phenol and amino nitrogen) may be attributed to the enhanced plant growth and biomass production (Kohler *et al*., 2007). These observations are in conformity with those from Aseri *et al.* (2008) who reported higher chlorophyll content in pomegranate inoculated with N_2-fixing bacteria. Mathur and Vyas (2000) reported enhanced synthesis of reducing sugars, free amino acids, soluble protein and chlorophyll in *Ziziphus mauritiana* seedlings inoculated with AM fungi.

Table 1: Effect of biofertilizer use on the growth, chlorophyll content and other metabolites of pomegranate cuttings (4 months after sowing)

Treatments	Branches (No. plant^{-1})	Leaf area (cm^2 plant^{-1})	Dry shoot weight (g plant^{-1})	Total chlorophyll (%)	Total phenols (%)	Amino nitrogen (%)	Reducing sugar (%)
A. chroococcum (Ac)	4	289.9	1.63	5.60	1.95	3.06	3.19
G. fasciculatum (Gf)	9	241.2	1.60	5.12	1.90	3.02	3.15
G. mosseae (Gm)	9	264.4	1.61	5.45	1.91	3.05	3.17
PO_4- solubilizing bacteria (PSB)	9	261.8	1.52	5.10	1.89	3.00	3.14
Ac + Gf	12	291.3	1.90	6.12	2.11	3.54	3.74
Ac + Gm	12	293.7	1.92	6.25	2.14	3.57	3.75
Ac + PSB	12	290.1	1.72	6.00	2.08	3.51	3.71
Control	4	180.4	1.39	5.05	1.78	2.68	2.01
$CD_{0.05}$	0.5	2.6	0.02	0.68	0.21	0.56	0.51

Effect on rhizosphere enzyme activities and mycorrhizal root colonization

Four months after inoculation, spore population of AM fungi and percent root colonization by AM fungi was enhanced by 103.6-183.9 and 267.8-444.5%, respectively, over control (Table 2). Uninoculated control also showed infection by mycorrhiza which could be attributed to the presence of native AM fungi in soil as the soil used was not sterilized. Dual inoculation resulted in maximum mycorrhizal root infection. The degree of root colonization and sporulation in rhizosphere soils varied significantly among different treatments. The degree of mycorrhizal colonization and sporulation was higher in dual inoculation with *A. chroococcum* + *G. mosseae* as compared to single inoculation treatments. This suggests that plant growth promoting rhizobacteria act as a helping tool for AM fungi for better establishment and functioning of symbiosis (Kohler *et al.*, 2007). Inoculation of soil with *Azotobacter* increased microflora population; whereas the addition of AM fungal inocula had synergistic effect on *Azotobacter*. The synergistic host response could mainly be due to the production of phytohormones or growth regulators produced by these microbes. It has been reported that spores of AM fungi seem to provide *Azotobacter* an operational base in the vicinity of roots and supply of carbon that increase the efficiency of both AM fungi and *Azotobacter* (Saxena and Tilak, 1994). There was a good agreement between AM fungal spore density and percent root colonization.

Table 2: Effect of biofertilizers on AM fungal spore density, mycorrhizal root colonization, rhizosphere enzyme activities and FDA hydrolysis, four months after inoculation.

Treatments	AM spores 50 g^{-1} soil (No.)	Mycorrhizal root colonization (%)	Dehydrogenase activity (µg g^{-1} soil)	Nitrogenase activity (mol C_2H_4 h^{-1})	Alkaline phosphatase activity (mol 100 g^{-1} soil)	FDA-hydrolysis (mol g^{-1} soil)
A. chroococcum (Ac)	236	72	8.26	392.0	13.2	2231
G. fasciculatum (Gf)	232	70	8.06	389.0	13.6	2223
G. mosseae (Gm)	233	71	8.21	390.0	13.5	2291
PO_4-solubilizing bacteria (PSB)	228	68	8.01	387.0	14.9	2211
Ac + Gf	315	96	9.15	410.5	16.9	2710
Ac + Gm	318	98	9.68	412.5	16.5	2772
Ac + PSB	313	96	9.25	411.5	16.8	2715
Control	112	18	6.25	217.0	9.6	1695
$CD_{0.05}$	3.65	2.04	0.56	1.87	0.64	27.1

Soil inoculation with biofertilizers significantly enhanced the enzyme activities like dehydrogenase, alkaline phosphatase and nitrogenase (N_2-ase) enzymes and hydrolysis of fluorescein diacetate in rhizosphere soils of pomegranate in comparison to uninoculated control plants (Table 2). The increase in dehydrogenase, alkaline phosphatase, nitrogenase and hydrolysis of fluorescein diacetate may be attributed mainly to the increase in rhizosphere microbial population as a consequence of inoculation treatments (Skujins, 1973; Aseri *et al*., 2008). The enhanced phosphatase activity improves phosphorus mobilization and thereby increases the biomass production (Tarafdar and Gharu, 2006). A significant enhancement in total nitrogen-fixing potential, as reflected by increased nitrogenase activity in rhizosphere soils, was observed. The estimation of these enzymatic activities provides early indication of changes in soil fertility, since they are related to mineralization of important nutrient elements such as N, P and C (Ceccanti *et al*., 1994).

Effect on nutrient uptake

Uptake of various nutrients was significantly higher in pomegranate upon inoculation with various beneficial microbes. In general, dual inoculation led to maximum uptake of N, P, K and micronutrients in pomegranate seedlings. In case of N dual inoculation treatment with *A. chroococcum* + *G. mosseae* or *A. chroococcum* + *G. fasciculatum* and in case of P dual inoculation treatment with *A. chroococcum* + PSB recorded higher uptake of nutrients (Table 3). Higher nutrient uptake was due to the synergistic effect of improved biomass and higher nutrient concentration in inoculated plants. Maximum N and P uptake was observed in dual inoculation treatments which may be due to the improvement in symbiotic N_2 fixation and phosphatase activities that enhanced phosphorus mobilization and subsequent P-uptake by mycorrhizal hypha. The results are in conformity with Singh and Sharma (1993) who reported enhanced N and P in sweet orange when inoculated with biofertilizers.

K, Cu, Zn, Mn and Fe were significantly higher in dual inoculation treatments with maximum in *A. chroococcum* + *G. mosseae* followed by *A. chroococcum* + PSB treatments. However, significant variations were noticed in the efficiency of different biofertilizers. Among nitrogen fixers and AM fungi, *A. chroococcum* and *G. mosseae*, respectively, enhanced maximum nutrient uptake as compared to control (Table 3). These results are in line with Patel *et al*. (2009) who reported enhanced uptake of K, Fe, Zn, Cu and Mn in sweet orange by the application of *Azotobacter* + AM fungi. The improvement could be attributed to the N-fixation ability of *Azotobacter*, better P mobilization and enhanced uptake of micro-nutrients due to AM fungi application.

Effect on plant height and yield

The inoculated plants exhibited significant enhancement in plant height and fruit yield (Table 4) with maximum increase in dual inoculation treatment of *A. chroococcum* + *G. mosseae*. Enhancement in plant

Table 3: Nutrient uptake (mg plant^{-1}) by pomegranate as influenced by inoculation with various biofertilizers (4 months after sowing)

Treatments	N	P	K	Cu	Zn	Mn	Fe
A. chroococcum (Ac)	23.7 (1.39)	3.4 (0.21)	18.1 (1.10)	0.026 (16.5)	0.15 (91.1)	0.26 (157)	0.22 (135)
G. fasciculatum (Gf)	22.0 (1.37)	4.8 (0.30)	18.6 (1.16)	0.030 (19.1)	0.14 (91.3)	0.25 (158)	0.24 (150)
G. mosseae (Gm)	22.5 (1.38)	5.6 (0.34)	20.3 (1.24)	0.027 (17.4)	0.15 (95.1)	0.26 (161)	0.26 (157)
PO_4-solubilizing bacteria (PSB)	22.8 (1.38)	3.1 (0.20)	18.6 (1.16)	0.026 (16.5)	0.14 (91.3)	0.26 (157)	0.23 (139)
Ac + Gf	26.5 (1.42)	6.1 (0.32)	22.4 (1.20)	0.036 (18.6)	0.16 (89.2)	0.28 (156)	0.29 (160)
Ac + Gm	27.0 (1.43)	6.4 (0.34)	22.8 (1.21)	0.037 (19.6)	0.17 (90.3)	0.29 (159)	0.30 (161)
Ac + PSB	26.5 (1.42)	7.4 (0.36)	22.6 (1.21)	0.036 (18.6)	0.16 (89.2)	0.29 (156)	0.31 (164)
Control	18.9 (1.36)	2.5 (0.18)	13.2 (0.95)	0.022 (16.3)	0.12 (86.9)	0.21 (152)	0.15 (106)
$CD_{0.05}$	0.29	0.12	0.06	0.001	0.02	0.01	0.02

The values of N, P and K in parenthesis indicate percent concentration in plants
The values of Cu, Zn, Mn and Fe in parenthesis indicate ppm concentration in plants

height and fruit yield was recorded right from the 1st year (2008-2009). Further, among the N_2-fixing bacteria and AM fungi *A. chroococcum* + *G. mosseae* induced improvement in parameters studied. However, overall maximum increase in these parameters was noticed in dual inoculation treatments followed by *A. chroococcum*. Higher accumulation of various metabolites, increased rhizosphere activity and better nutrient uptake appears to have favourably influenced the growth of transplanted pomegranate in field conditions. The growth trend was almost same in all the treatments as observed under nursery conditions. Increased plant growth and fruit yield due to biofertilizers under field conditions have previously been reported in banana and tomato by Jeeva *et al.* (1988) and Kholer *et al.* (2007), respectively.

Table 4: Effect of inoculation with various biofertilizers on plant height and fruit yield of pomegranate grown in field conditions

Treatments	Plant height (cm)	Fruit yield (kg plant^{-1})
A. chroococcum (Ac)	124.17	26.9
G. fasciculatum (Gf)	113.47	21.2
G. mosseae (Gm)	119.24	24.4
PO_4-solubilizing bacteria (PSB)	108.48	20.8
Ac + Gf	158.52	31.3
Ac + Gm	198.45	34.7
Ac + PSB	176.43	29.1
Control	76.37	10.4
$CD_{0.05}$	2.46	3.49

Conclusion: Based on the present investigation it may be concluded that the use of biofertilizer technology may play a vital role in early establishment and survival of pomegranate (*Punica granatum*) in stressed soils. The pre-inoculation of nursery seedlings with selected N_2-fixing bacteria and AM fungi alone or in combination may help in the production of vigorous plants capable to survive and thrive in stress soils.

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