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Evaluation of plant beneficial microbes for bio-hardening of *in-vitro* raised pomegranate saplings

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ABSTRACT

Five beneficial microbial agents either alone or in combinations were used to inoculate one year old *in-vitro* raised pomegranate plants of cv. Bhagawa for further utilization of these microbes as bio-hardening agents. A pot culture experiment was initiated comprising 15 treatments each having three replications. The growth, physiological and biochemical parameters of the inoculated and control plants were recorded at 90 days after inoculation. Microbes and microbial formulations were evaluated for their further utilization as bio-priming agents in *in-vitro* raised pomegranate plants. Plants inoculated with *Aspergillus niger* strain AN27 *(Asp)* + *Penicillium pinophilum* registered significant increase in plant height (225.00 cm) and plant spread E-W (198.33 cm) as compared to control. *Asp* + *Pseudomonas fluorescens* inoculated plants performed significantly better with respect to total chlorophyll (1.84 mg/g FW), SPAD value (41.23) and total phenol content (0.81 mg/g FW) as compared to non-inoculated plants. Plants inoculated with AMF (*Glomus intraradices*) + *T. viridae and T. harzianum* was at par with *Asp* + *P. fluorescens* in total chlorophyll content (1.71 mg/g FW) and SPAD value of leaves (36.10). Plants inoculated with AMF in combination with *P. pinophilum* (T10), *P. fluorescens* (T11) and *T. viridae and T. harzianum* (T12) registered higher photosynthesis as compared to control and many other treatments. Microbial treatments were found effective in improving most of the morphological, physiological and biochemical attributes of inoculated pomegranate plants.

Key words: Bio-hardening, Micro-propagation, Plant beneficial microbes, Pomegranate

Pomegranate (*Punica granatum* L.) is one of the most remunerative fruit crops that can thrive well under arid and semi-arid agro ecosystem. Pomegranate has become the choicest fruit crop for cultivation in semi-arid regions of India and the world due to its unique adaptability, hardy nature, less irrigation water requirement, good response to high tech-horticultural practices, considerable demand in cosmetic and pharmaceutical industry, higher returns on investment than other crops of dry regions, immense therapeutic values, high global demand for table and processed products and immense export potential (Maity *et al*. 2014, Sharma *et al*. 2014). Since, last one decade, India is experiencing a pomegranate revolution and currently occupying *numero uno* position in the world with about 2.62 lakh ha. area producing about 30.3 lakh tonnes of pomegranate. (http://nhb.gov.in). The rapid expansion of pomegranate in recent times without the availability of elite and healthy saplings has led to the use of unauthentic saplings carrying latent infections resulting into the spread of bacterial blight disease through infected planting material (Singh *et al*. 2014; Singh *et al.* 2020). Recently, ICAR-NRCP has developed a micropropagation protocol for production of disease-free elite planting material of pomegranate (Singh *et al*. 2013). Though, the *in-vitro* propagation method helps in obtaining large number of high-quality, uniform and disease free plants. But, proper the acclimatization or hardening of *in-vitro* raised plants is critical as plants are susceptible to various stress after transfer from *in-vitro* to *ex-vitro* greenhouse conditions (Schubert *et al.* 1990, Hazarika 2003, Singh *et al*. 2012, Singh *et al*. 2016).

Hence, the *ex-vitro* acclimatization of the tissue culture raised plants using plant beneficial microbes can be carried out for improved functioning. These plant beneficial microbes are efficient in significantly enhancing soil exploration capacity of the host and increasing nutrient availability by bringing them in readily available form from fixed state thereby improving field survival, photosynthesis, host plant resistance and nutrient uptake by the host plant (Paula *et al.* 1993, Puthur *et al.* 1998, Rupnawar and Navale 2000, Sen 2000, Krishna *et al.* 2006, Aseri 2008, Singh *et al.* 2016).

MATERIALS AND METHODS

Present study was carried out during 2017-18 and 2018-

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19 in the experimental polyhouse of the ICAR- National Research Centre on Pomegranate, Solapur, India, located at geographical coordinates of 17°68′ N latitude, 75°91′ E longitude and at 483.5 m altitude from mean sea level. One year old *in-vitro* raised pomegranate plants of cultivar Bhagawa were used. Before the beginning of experiment, 1 year old *in-vitro* raised plants were re-potted in the 20 kg capacity cemented pots having autoclaved sterile potting mixture (sand : soil : FYM, 2:1:1). Plants were maintained in 35 % shade under shade net house and were watered on alternate days with tap water. The commercial products namely, Kalisena® (*Aspergillus niger* strain AN27) and Josh ultra® (AMF predominantly (*Glomus intraradices*) were used. The AMF was dissolved in distilled water (15 g/100 ml/plant) whereas *A. niger* was mixed with PDB (20 g/100 ml/plant) one day before mixing into the pot mixture. The pure cultures of *Pseudomonas fluorescens, Penicillium pinophilum* and *Trichoderma viridae* + *T. harzianum* maintained at ICAR-NRCP were used. These organisms were sub-cultured on nutrient glucose agar media (NGA) and potato dextrose agar media (PDA), respectively and were incubated until satisfactory growth on the medium. Subsequently, the microbes were inoculated into the respective culture broths, i.e. NGB and PDB just before mixing into pot mixture. The above treatments were inoculated in the potting mixture twice, at the interval of 15 days.

Microbial treatments

Treat.	Strain
T1	*Aspergillus niger* (strain AN27)
T2	AMF (*Glomus intraradices*)
T3	*Penicillium pinophilum*
T4	*Pseudomonas fluorescens*
T5	*Trichoderma viridae* + *T. harzianum*
T6	*A. niger* + AMF
T7	*A. niger* + *P. pinophilum*
T8	*A. niger* + *P. fluorescens*
T9	*A. niger* + *T. viridae* & *T. harzianum*
T10	AMF + *P. pinophilum*
T11	AMF + *P. fluorescens*
T12	AMF + *T. viridae* & *T. harzianum*
T13	*P. pinophilum* + *P. fluorescens* + *T. viridae* & *T. harzianum*
T14	*A. niger* + AMF + *P. pinophilum* + *P. fluorescens* + *T. viridae* and *T. harzianum*
T15	Control

Height of potted Bhagawa plants was measured at 90 days after inoculation (DAI) using graduated hollow pipe. Average length was expressed in cm. Plant spread was measured in East-West and North- South direction in cm. Stem girth of plants were measured using Vernier Caliper (Baker) and average girth of the main stem was expressed in mm. Fresh weight of shoots and roots were measured immediately after completion of non-destructive observations. Shoots and roots were washed with tap water to remove the adhering dust and potting mixture. Thereafter the excess water on the root surface was removed by gentle swabbing with blotting paper and fresh weights were noted immediately using electronic balance.

The Relative water content (RWC) in leaves was determined using method suggested by Weatherley (1950).

$$\text{RWC (\%)} = \frac{\text{Fresh weight} - \text{Oven dry weight}}{\text{Turgid weight} - \text{Oven dry weight}} \times 100$$

Three mature leaves were collected from each plant and the rate of photosynthesis was measured using LI-6400 Portable Photosynthesis System (LI-COR). Average rate of photosynthesis was expressed in µmol CO_2 / m^2/ s.

The SPAD-502 Chlorophyll Meter (KONICA MINOLTA) had been used to instantly measure greenness of leaves.

The method proposed by Malik and Singh (1980) was used for quantification of total phenols content and expressed as catechol equivalent. Instead of buds, shoot tips along with freshly emerged leaves were used.

The chlorophyll content (chlorophyll a, b and total chlorophyll) of the leaves was analyzed at 90 days after inoculation following the method as suggested by Barnes *et al.* (1992). Fully mature open leaves were chosen as the experimental sample for chlorophyll estimation.

Reducing sugars were estimated at 90 DAI following the method suggested by Somogyi (1951). Absorbance corresponds to 0.1ml of test = x mg of glucose.

$$\text{Reducing sugars (\%)} = \frac{x}{0.1} \times 100$$

Proline was estimated using Acid ninhydrin regent method (Thimmaiah 2006).

$$\mu \text{ moles proline/g tissue} = \frac{\mu\text{g proline/ml} \times \text{ml toluene}}{115.5} \times 5$$

There were 15 treatments and each treatment was having three replications. The data obtained from the experiment were subjected to analysis of variance (ANOVA) which was done by using freely available online software WASP 2.0 (Web-based Agri Stat Package) developed by ICAR-CCARI, Goa.

RESULTS AND DISCUSSION

Different bioagents have been employed to improve the morphological, physiological and biochemical functioning of *in vitro* raised pomegranate plants of cv. Bhagawa. In total five microbial agents and their combinations were used for biopriming of *in vitro* raised plants by incorporating them in the rhizosphere.

Effect of microbe(s) inoculation on growth parameters of the host plant: Plants inoculated with combination of *Aspergillus niger* (strain AN 27) + *Penicillium pinophilum*

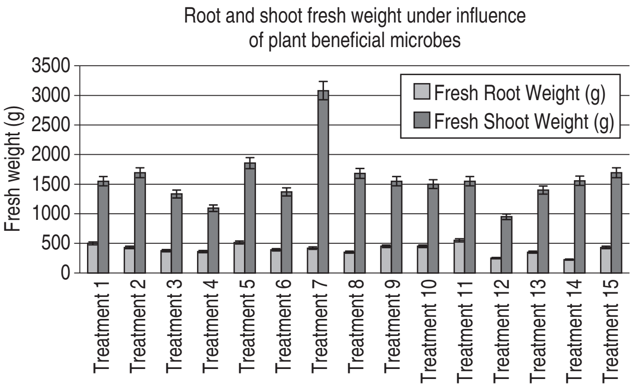

Fig 1 Root and shoot fresh weight under influence of plant beneficial microbes.

i.e. treatment 7 showed significantly better plant height and canopy spread in East-West direction (225.00 and 198.33 cm, respectively) than control (131.67, 93.33 cm, respectively). Treatment 5 (195.00 cm), treatment 13 (190.00 cm), and Treatment 8 (186.67 cm) were at par with treatment 7 as far as plant height was concerned and treatment 8 (175.00 cm), treatment 9 (158.33 cm), treatment 5 (156.67 cm), treatment 11 and treatment 6 (150.00 cm) for canopy spread (Table 1). However, stem diameter did not vary significantly across the treatments. Plants inoculated with *A. niger* + *P. pinophilum* (treatment 7) registered the maximum shoot fresh weight, however, root fresh weight was found highest in plants inoculated with AMF + *P. fluorescens* followed by *Aspergillus niger* and *Trichoderma viridae* + *T. harzianum* inoculated plants (Fig 1).

The higher biomass production could be the result of improved nutrient and water uptake, and probably the enhanced photosynthetic rate of microbe inoculated plants (Mathur and Vyas 1999; Vidal *et al.* 1999) and higher soil exploration capacity of host plants through mycelia of fungal microbes (Krishna *et al.* 2006). The increased biomass and dry matter in microbe inoculated plants of pomegranate was earlier observed by Rupanawar and Navale (2000). Maity *et al*. (2014) reported increase in shoot, root and total dry biomass of pomegranate in *Penicillium pinophilum* inoculated plants as compared to non inoculated control, they suggested that the plant growth promotion was related to enhanced 'K' and 'P' solubilization and uptake plus the release of plant growth promoting substances by the *P. pinophilum*.

Effect of microbe(s) inoculation on physiological parameters of the host plant: Plants treated with AMF + *P. fluorescens* (26.94 µmol $CO_2/m^2/s$), AMF + *P. pinophilum* (26.65 µmol $CO_2/m^2/s$) and AMF + *T. viridae* & *T. harzianum* (26.36 µmol $CO_2/m^2/s$)) registered significantly better photosynthetic rate as compared to untreated plants (13.907 µmol $CO_2/m^2/s$). Plants inoculated with Treatment 8 (24.07 µmol $CO_2/m^2/s$), Treatment 13 (23.12 µmol $CO_2/m^2/s$), Treatment 1 (20.09 µmol $CO_2/m^2/s$) and Treatment 7 (19.65 µmol $CO_2/m^2/s$) were also at par with above mentioned treatments (Table 1). However, RLWC varied non-significantly across the treatments and non-inoculated control (Table 1). The SPAD rate of plants inoculated with treatment 8 (41.23) registered significantly better SAPD value than the control plants (24.53). Plant inoculated with treatment 12 (36.10) also performed at par with treatment 8 (Table 1).

Increase in photosynthetic rate might be attributed to improve in synthesis of chlorophyll, increase in leaf area and better uptake of nutrients and water (Bavaresco *et al*. 1995,

Table 1 Effect of plant beneficial microbes on growth, physiological and biochemical attributes of host plant

Treatment	Plant height (cm)	Plant spread E-W (cm)	Plant spread N-S (cm)	Stem dia. (mm)	RLWC (%)	SPAD value	Photosynthetic rate (µmol $CO_2/m^2/s$)	Reducing sugars (%)	Leaf Proline content (µmol/g)	Total phenols (mg/g FW)
T1	170.00	101.67	110.00	21.84	84.41	30.80	20.09	0.64	4.32	0.79
T2	168.33	128.33	123.33	20.41	88.79	29.97	15.30	0.89	0.96	0.78
T3	185.00	116.67	111.67	20.61	87.26	26.50	12.27	0.72	0.90	0.77
T4	183.33	113.33	101.67	21.50	82.51	33.40	14.38	0.67	1.08	0.77
T5	195.00	156.67	131.67	24.61	85.34	26.03	14.80	0.52	1.98	0.76
T6	160.00	150.00	126.67	26.91	84.21	31.07	14.50	0.73	3.84	0.76
T7	225.00	198.33	175.00	27.80	85.09	26.77	19.65	0.95	2.16	0.76
T8	186.67	175.00	135.00	27.20	88.42	41.23	24.07	0.87	1.80	0.81
T9	180.00	158.33	146.67	22.03	88.79	26.67	17.02	0.82	0.96	0.79
T10	170.00	148.33	143.33	22.00	84.54	31.70	26.65	0.67	2.28	0.80
T11	168.33	150.00	130.00	23.22	87.03	25.53	26.94	0.71	2.05	0.80
T12	171.67	115.00	106.67	20.13	88.63	36.10	26.36	1.14	3.60	0.79
T13	190.00	135.00	141.67	23.42	86.60	24.67	23.12	1.17	2.36	0.79
T14	183.33	141.67	126.67	26.27	89.10	30.63	14.85	1.09	4.7	0.78
T15	131.67	93.33	96.67	19.71	83.75	24.53	13.91	0.55	1.80	0.75
CD (P=0.05)	39.18	49.01	NS	NS	NS	7.77	7.41	0.41	NS	0.03

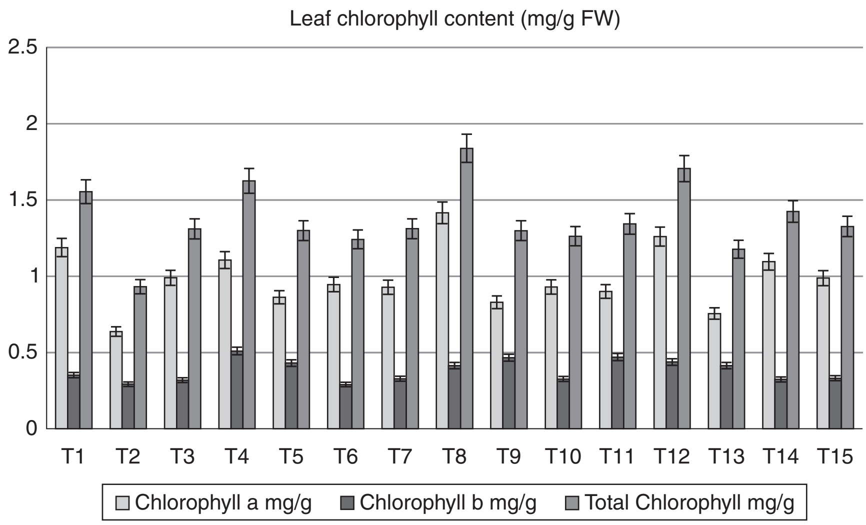

Fig 2 Leaf chlorophyll as influenced by plant beneficial microbes.

Singh *et al.* 2012, Singh *et al*. 2016). The results obtained by SPAD chlorophyll meter clearly showed the superiority of all the treated plants over the control. These values are indirectly used to predict the physiological condition and chlorophyll content of the leaves and nitrogen status of the plants. The increased RWC can also be attributed to the improved water transport because of reduced root resistance owing to increased uptake of phosphorus and other nutrients by AMF and other microbe(s) inoculated plants (Aseri 2008, Singh *et al.* 2012, Singh *et al*. 2016). Increased nutrient uptake particularly P and K by plants inoculated with *P. pinophilum* might have played role in enhancing relative leaf moisture content, SPAD value and photosynthetic rate of plant, besides, K has substantial effect on enzyme activation, protein synthesis, photosynthesis, stomatal movement and water relation in plants (Maity *et al*. 2014).

Effect of microbe(s) inoculation on biochemical parameters of the host plant: Treatment 8 (0.81 mg/g FW) treated plants registered significantly better total phenolic content than non-inoculated control plants (0.75 mg/g FW). Seven other treatments were at par with treatment 8 namely, Treatment 10, Treatment 11, Treatment 12, Treatment 13, Treatment 9, Treatment 1 Treatment 2 and Treatment 14. These above treatments were significantly better than other treatments and control (Table 1). Total leaf chlorophyll content was also significantly better in plants treated with treatment 8 (1.84 mg/g FW) as compared to control (0.93 mg/g FW). Plants treated with Treatment 12 (1.71 mg/g FW), Treatment 4 (1.63 mg/g FW) and Treatment 1 (1.55 mg/g FW) were at par with treatment 8 (Table 1 and Fig 2).

Plants inoculated with *P. pinophilum* + *P. fluorescens* + *T. viridae & T. harzianum, i.e.* treatment 13 (1.17%) produced significantly higher leaf reducing sugars than non-inoculated control plants (0.55%). Plants treated with Treatment 12 (1.14%), Treatment 14 (1.09%), Treatment 7 (0.95%), Treatment 2 (0.89%), Treatment 8 (0.87%) and Treatment 9 (0.82%) also registered at par leaf reducing sugar content (Table 1).

Phenolic content increase was attributed to the enhanced polyphenol oxidase activity in mycorrhized plantlets (Mathur and Vyas 1999). Inoculation with AMF resulted in accumulation of different biochemicals in the plantlet such as chlorophyll, carotenoids, proline, phenol, polyphenol oxidase and nitrate reductase (Krishna *et al.* 2005). Most of these plant beneficial microbes secrete organic acids like citric, oxalic and tartaric acids which facilitate the weathering of minerals by directly dissolving K and P from rocks or through the formation of metal-organic complexes or by forming chelate to bring them into solution thus facilitate the uptake by plants (Maity *et al*. 2014). The increase in chlorophyll content of leaves was due to better uptake of plant nutrients, i.e. Mg, Fe and Cu which are essential for chlorophyll synthesis (Krishna *et al*. 2006). Therefore, our results provided evidence of the benefits of plant beneficial microbes for biohardening of *in vitro* raised plantlets to improve their field performance. The plant beneficial microbes have immense potentiality to improve growth, physiological and biochemical performance of *in vitro* raised pomegranate saplings. In the present investigation, *Aspergillus niger* in combination with *Penicillium pinophilum* and *Pseudomonas fluorescens* were found to be a good consortium of microbes to improve performance of plants, however, AMF (*Glomus intraradices*) has also significantly impacted plant performance either alone or in combination with other microbes. These plants beneficial microbes may be effectively utilized as biohardening agents to improve field performance of *in vitro* raised pomegranate saplings.

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