Indian Journal of Agricultural Sciences **86** (1): 132–6, January 2016/Article
https://doi.org/10.56093/ijas.v86i1.55252

Bio-hardening of *in-vitro* raised plants of Bhagwa pomegranate (*Punica granatum*)

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Received: 19 January 2015; Accepted: 28 September 2015

ABSTRACT

A pot culture experiment on bio-hardening of the *in-vitro* raised plants of Bhagwa pomegranate (*Punica granatum* L.) was conducted to find out the effect of two commercially available bio-formulations containing Arbuscular Mycorrhizal Fungi (AMF) and *Aspergillus niger* strain AN-27. Observations on root colonization, population dynamics of microbes in rhizospheric soil, growth, physiological and biochemical parameters of biohardened plants were recorded at 180 days after inoculation. Results revealed that AMF colonization in roots of *in-vitro* raised pomegranate plants was found at par in plants inoculated with AMF (71.12%) and AMF + Asp (65.00%) the root colonization in these two treatments was significantly higher than Contral (10.00%). Population of *Aspergillus niger* in the rhizospheric soil was found significantly higher in soil inoculated with *Aspergillus niger* strain AN-27 (6×10^4 cfu/g of soil) as compared to non-inoculated control (2×10^4 cfu/g of soil). Significant improvement in plant height (27.0% increase), shoot fresh weight (24.8% increase), root fresh (23.3% increase) and dry weight (15.7% increase) was observed in bio-hardened plants than untreated ones. Physiological processes were also significantly improved by bio-hardening as AMF and AMF + Asp treated plants registered significantly better RWC (92.34 and 91.74 %, respectively) and photosynthesis (12.69 and 12.78 µmol $CO_2m^{-2}s^{-1}$, respectively) as compared to control (87.76 % and 9.07 µmol $CO_2m^{-2}s^{-1}$, respectively) and only Asp treated plants (89.99% and 9.70 µmol $CO_2m^{-2}s^{-1}$, respectively). AMF inoculated and AMF + Asp treated plants registered significantly higher total leaf chlorophyll (2.97 and 3.14 mg/g fresh weight, respectively) and total phenolic content (53.00 and 52.50 mg catechol equivalent/ 100 g fresh wt., respectively) than the control and only Asp treated plants.

Key words: Arbuscular mycorrhizal fungi, *Aspergillus niger* strain AN-27 Bio-hardening, Pomegranate

Healthy and disease-free planting material is an important factor for sustainable production of pomegranate (*Punica granatum* L.). Traditional propagation methods need to be supplemented with modern propagation techniques to fulfill the constantly increasing requirement of elite planting material. This ever increasing demand of quality planting material of pomegranate can only be met when conventional and non-conventional methods of pomegranate propagation are exploited at commercial scale with need based modifications involving modern propagation technologies. Recently, ICAR-NRCP has developed a protocol for *in-vitro* propagation of pomegranate for production of disease free elite planting material (Singh *et al*. 2013). But endeavor is still under progress to produce bio-hardened and better field performing pomegranate plants using tissue culture technique in an economic manner. *In-vitro* culture methods facilitate obtaining large number of high-quality genetically uniform and disease free plants in less time. But, acclimatization of *in-vitro* raised plants to *ex-vitro* conditions is very crucial and an important determinant of subsequent field establishment of these plants (Singh *et al*. 2012). The micropropagated plants are susceptible to various stresses after transfer from *in-vitro* to *ex-vitro* green house conditions because of the sudden environmental changes and certain incompleteness of physiological development of the plant system. These *in-vitro* raised plantlets have poorly formed and weak root system (Hazarika 2003), unfavourable nutritional and environmental conditions (Schubert *et al*. 1990), poorly developed cuticle and/or non-functional stomata (Hazarika 2003). Development of robust root system in the key element for field establishment of tissue cultured saplings. The *ex-vitro* performance of tissue culture raised plantlets can effectively be improved by utilizing plant beneficial microbes or bioagents like arbuscular mycorrhizal fungi (AMF) and *Aspergillus niger* strain AN-27 in the hardening process (Rupnawar and Navale 2000 and Mondal *et al.*

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2000). Bio-hardening of *in-vitro* raised plants utilizing plant beneficial microbes and placing their formulations in rhizosphere and phyllosphere of *in-vitro* raised plants, ascertain the improved performance by virtue of improved morphological, physiological and biochemical functioning bio-hardened plants. AMF develop a symbiotic relationship with the plant that enhances the development of the root. *Aspergillus niger* strain AN-27 can be used as a biofertilizer as well as a biocontrol agent hence, using it for biohardening of pomegranate saplings prior to planting in field-as well as field application of this bioformulation-will ensure vigorous plants with good yield (Sen 2000).

Therefore, a study was undertaken with the objective of evaluation of performance of tissue cultured pomegranate saplings by *ex-vitro* bio-hardening.

MATERIALS AND METHODS

The study was carried out in the experimental polyhouse of the ICAR-National Research Centre on Pomegranate, Solapur, India, located at 17°68' N latitude, 75°91'E longitude and 457 m altitude from mean sea level, during the period of 2013-14. *In-vitro* raised plantlets of pomegranate cultivar Bhagawa (30 days old rooted plantlets) were used for bio-hardening. Plantlets were maintained in a polyhouse with temperature range of 27± 2ºC. Humidity was maintained at 85% using fogger (Netafim^{TM}). Plantlets were watered on alternate days.

In-vitro propagation protocol was standardized using nodal explants collected from healthy mature plants of cultivar Bhagwa maintained in the Experimental Orchard at ICAR-National Research Centre on Pomegranate, Solapur, India.

The commercial products of AMF (*Rhizophagus intraradices* syn *Glomus intraradices*) and *Aspergillus niger* (strain AN 27) were used containing 60000 infectious propagules per kg of product and 3×10^5cfu per g of product, respectively.

Thirty days old rooted plantlets from sterile hardening medium (cocopeat: perlite: vermiculite, 1:1:1) were transferred to presterilized potting mixture (sand: soil: FYM, 1:1:1). At the time of transfer, 5 g of commercial formulation of AMF containing 300 infectious propagules per kg of potting mixture was placed in treatments T1(only AMF inoculated plants) and T3 (AMF + *Aspergillus niger* strain AN 27 treated plants) near the root zone of the *in-vitro* raised plants. *Aspergillus niger* strain AN 27 was multiplied on decomposed FYM @ 1 kg *Aspergillus niger* strain AN 27 along with the carrier material per 500 kg of FYM and kept under shade for 10 days with sprinkling of water and racking after every 2 days, from this mixture 30 g per kg of potting mixture was placed near the root zone in treatments T2 (*Aspergillus niger* strain AN 27 treated plants) and T3.

Observations on various growth, physiological and biochemical parameters were recorded to study the effect of different bioinoculants on inoculated plantlets. The data were recorded at 180 days after treatment (DAT).

Observations were recorded on root colonization with AMF, *Aspergillus niger* population in the rhizosphere soil, plant height, shoot fresh and dry weight and root fresh and dry weight. Plantlets at 180 DAT were used for assessing per cent root colonization by staining method as suggested by Phillips and Hayman (1970). For assessment of per cent root colonization, root segments (1 cm long from the elongation region) were selected at random from a stained sample and mounted on microscopic slides in a group of 10 and were observed under a microscope (Model Eclipse 90i, Nikon, Japan) under 1000 × magnification. Extent of root colonization was assessed in 10 portions per root segment. The average values were expressed as a percentage of root length.

The population count of *Aspergillus niger* was assessed using soil dilution plate technique (Dhingra and Sinclair 1986). Rhizosphere soil from root surface of uprooted pomegranate plants was collected in sterile polythene bags and mixed thoroughly. 10g soil was added to 90 ml sterile distilled water and serially diluted to 10^3 dilution. 1 ml of the 10^{-3} dilution was spread on the surface of pre-poured Potato Dextrose Agar media in 90 mm Petri plates. Each treatment was replicated five times. Plates were incubated at 28+1ºC and *Aspergillus niger* colonies were counted on third day of incubation. The colony forming units (cfu) per gram were calculated using the formula:

Number of cfu/g = No. of colonies per plate × 10^3

Five plants in each replication were randomly selected and the lengths of plants were measured at 180 DAT from the soil level. Average length was expressed in centimeter. Fresh weight of shoots was measured just after sampling. Samples for dry matter determination were put in brown paper bags and kept in a hot air oven at 70°C till they showed no change in weight for 2 consecutive days. Roots were washed with tap water to remove the adhering potting mixture. Thereafter, the excess water on the root surface was removed by gentle swabbing with blotting paper. Fresh weights were noted immediately, while for determination of dry weights, procedure mentioned earlier for shoot dry weight was followed.

The relative water content (RWC) of the recently matured leaf was determined using the method suggested by Weatherley (1950). Five recently matured and fully opened leaves were randomly collected. Fresh weight of these leaves were measured and then floated overnight on distilled water in Pertidish. These discs were then surface dried by placing them in between 2 sheets of Whatman No. 1 filter paper and saturated weight of these discs were recorded. After that, the samples were dried in an oven at 70ºC till they showed no change in their weight after two consecutive drying. The dry weight of the sample was then recorded.

The RWC was then estimated using the formula:

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

Photosynthetic rate was measured by using (Portable Photosynthesis System, LI-COR 6400) by selecting five mature leaves on each plant and expressed as µmolCO_2/ m^2/s.

Among biochemical parameters total phenolic content, chlorophyll a, b and total chlorophyll were estimated.

The method proposed by Malik and Singh (1980) was employed for quantification of total phenols using LABINDIA, UV 3000+ spectrophotometer. Shoot tips along with a pair of freshly emerged leaves were taken instead of buds.

Chlorophyll 'a', 'b' and 'total leaf chlorophyll' were determined at 180 days after inoculation following the method as suggested by Barnes *et al*. (1992). Fully mature open leaves were chosen as the sample for chlorophyll determination. Accurately weighed 0.5 g of clean leaf sample was immersed in 10 ml of dimethyl sulphoxide (DMSO) AR. Each sample was incubated at 70 °C for 4 h in a hot air oven. It was then taken out and 1 ml of the solution was diluted to 5 ml with DMSO and the sample was read on a spectrophotometer at 645 and 663 nm using pure DMSO as blank, total chlorophyll was calculated according to the following formula:

$$\text{Total chlorophyll (mg/g)} = \frac{(20.20\times O.D.645)+(8.02\times O.D.663)\times \text{Volume}\times \text{Dilution}}{1000\times \text{Weight of sample}}$$

All experiments were set up in a completely randomized design with five replications in each treatment and each replication having five plantlets. The analysis of variance was carried out to detect overall significant differences among the treatment means. Percentages were subjected to arc sine % transformation in radians before ANOVA. The coefficient of variation for various traits was also calculated. The analysis was carried out using Web Based Agricultural Statistics Software Package (WASP 2.0) developed by ICAR-Central Coastal Agricultural Research Institute, Goa.

RESULTS AND DISCUSSION

Root colonization and microbial population in rhizosphere

Colonization of roots of *in-vitro* raised pomegranate plants with Arbuscular Mycorrhizal Fungi (AMF) was found at par in plants inoculated with AMF (71.12 %) and plants inoculated with AMF + *Aspergillus niger* (65.00%) (Table 1). Population of *Aspergillus niger* strain AN-27 in the rhizosphere soil was significantly higher in soil inoculated with *Aspergillus niger* (6×10^4cfu/g of soil) as compared to non-inoculated control (2×10^4cfu/g of soil). The degree of infection by AMF is dependent on post-inoculation duration and the species of arbuscular mycorrhizal fungi. Singh *et al*. (2012) reported that the rate of infection rose to two folds between 60 and 90 days after infection of *in-vitro* raised pomegranate pomegranate plants of cv. G-137. Application of *Aspergillus niger* strain AN-27 has been reported to reduce soil borne pathogens in several horticultural crops by increasing population of *A. niger* and antagonizing the pathogens through, lysis or production of inhibitory metabolites as well having growth promoting and yield enhancing properties (Sen 2000).

Influence of bio-hardening on growth parameters

Plant height, shoot fresh and dry weight and root fresh and dry weight were recorded for establishing the superiority of bio-hardened tissue culture raised plants over non bio-hardened plants. Except for shoot dry weight, which was found non-significant among various treatments all other growth parameters were significantly influenced by bio-hardening agents (Table 1). As the result of bio-hardening, shoot fresh weight was found significantly higher in AMF treated (196.03 g), Asp treated plants (183.23 g) and AMF + Asp treated plants (184.23 g) as compared to non-inoculated control (157.09 g). Similarly, root fresh and dry weights significantly increased in AMF (61.44 and 16.92 g, respectively) Asp (60.40 and 16.04 g, respectively) and AMF + Asp treated plants (58.44 and 16.30g, respectively) as compared to non-inoculated control (49.84 and 14.63g, respectively).

Significant improvement in plant height (27.0% increase), shoot fresh weight (24.8 % increase), root fresh (23.3% increase) and dry weight (15.7% increase) were

Table 1 Effect of bio-hardening on growth, physiological and biochemical attributes of tissue culture raised pomegranate plants

Treat-ment	Root coloniz ation with AMF (%)	Colony forming unit /g of rhizospheric soil	Plant height (cm)	Shoot fresh wt. (g)	Shoot dry wt. (g)	Root fresh wt. (g)	Root dry wt.(g)	Leaf relative water content (%)	Photosynthesis (µmol $CO_2m^{-2}s^{-1}$)	Total phenol content (mg catechol equivalent /100 g Fresh wt.)
T_0	10.00 (18.44)*	2.0×10^3	110.69	157.09	61.13	49.84	14.63	87.76 (69.54)	9.07	29.50
T_1	71.12 (57.68)	1.6×10^3	140.63	196.03	74.25	61.44	16.92	92.34 (73.96)	12.69	52.50
T_2	12.50 (20.46)	6.1×10^3	130.38	183.23	73.14	60.40	16.04	89.99 (71.60)	9.70	42.00
T_3	65.00 (53.85)	4.8×10^3	134.63	184.23	74.94	58.44	16.30	91.74 (73.31)	12.78	53.00
P=0.05	6.36	1.5×10^3	15.25	23.81	NS	7.62	1.53	1.97	3.02	12.34

T_0- Control, T_1- AMF, T_2- *Aspergillus niger* (strain AN 27), T_3 - AMF + *Aspergillus niger* (strain AN 27). *transformed values

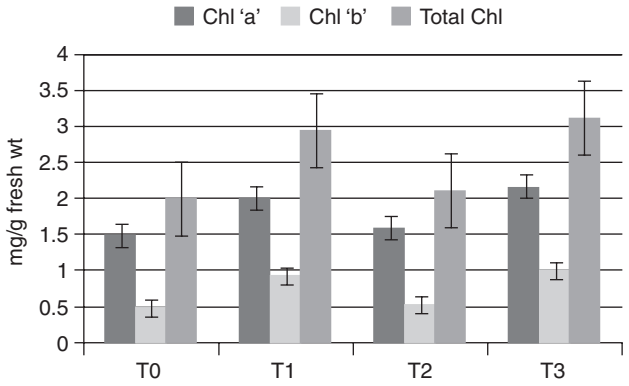

Fig 1 Effect of bio-hardening on leaf chlorophyll content.

found as the result of bio-hardening. Previously, Rupnawar and Navale (2000), Aseri *et al*. (2008), Singh *et al*. (2012) and Mondal *et al.* (2000) also observed increased plant height, fresh and dry biomass of plants as the result of mycorrhiza and *Aspergillus niger* strain AN-27 treatment/ inoculation.

Mathur and Vyas (1999) came up with the conclusion that the higher biomass production could be attributed to the improved nutrient uptake, possibly by an enhanced photosynthetic rate.

Physiological and biochemical parameters as influenced by bio-hardening

Physiological activities were also significantly improved by biohardening and to establish this fact two important plant physiological activities namely, Leaf Relative Water Content and Photosynthesis were measured. AMF and AMF + Asp treated plants registered significantly better RWC (92.34 and 91.74 %, respectively) and higher photosynthesis (12.69 and 12.78 µmol $CO_2m^{-2}s^{-1}$, respectively) as compared to control and only Asp treated plants. However, performance of AMF inoculated plants either alone or in combination of *Aspergillus niger* excelled significantly as compared to control and only *Aspergillus niger* treated plants (Table 1). The increased RWC could be attributed to improved water uptake by increasing root exploration of soil volume, improved plant nutrition and/or regulating stomatal opening through hormonal biosynthesis (Reid 1979, Krishna *et al*. 2006, Singh *et al*. 2012). The increased RWC can also be attributed to the improved water transport because of reduced root resistance owing to increased uptake of phosphorus by AMF (Safir *et al*. 1972).

High photosynthetic activity of AMF inoculated and AMF + *Aspergillus niger* treated plants may be due to higher leaf chlorophyll content of plants under the influence of these two treatments as compared to control and only Asp treated plants (Fig 1). Earlier, Mathur and Vyas (1995) and Yang *et al*. (2014) also found the similar results and explained the enhanced chlorophyll synthesis as the reason for increased photosynthesis.

AMF inoculated and AMF + *Aspergillus niger* treated plants registered significantly higher total leaf chlorophyll (2.97 and 3.14 mg/g fresh weight, respectively) and total phenol content (53.00 and 52.50 mg Catechol equivalent/ 100 g fresh wt., respectively) than the control and only Asp treated plants. The increase in the chlorophyll content of leaves could be attributed to enhanced uptake of Mg, Cu and Fe, which are essential for chlorophyll synthesis (Krishna *et al*. 2005).

Bio-hardened and better field performing tissue cultured plants ensure disease free planting material which can satisfactorily tolerate biotic and abiotic stresses under field conditions. AMF either alone or in combination with *Aspergillus niger* strain AN-27 can effectively be used as biohardening agents to improve growth, physiological and biochemical activities of tissue cultured plant and hence their subsequent field performance.

REFERENCES

Aseri G K, Jain N, Panwar J, Rao A V and Meghwal P R. 2008.Biofertilizers improve plant growth, fruit yield, nutrition, metabolism and rhizosphere enzyme activities of pomegranate (*Punica granatum* L.) in Indian Thar Desert. *Scientia Horticulturae* **117**(2): 130–5.

Barnes J D, Balaguer L, Maurigue E, Elvira S and Davison A W. 1992. A reappraisal of the use of DMSO for the extraction and determination of chlorophyll 'a' and 'b' in lichens and higher plants. *Environmental and Experimental Botany* **32**(2): 87–99.

Dhingra O D and Sinclair J B. 1986. *Basic Plant Pathology Methods*, p 355 CRC Press, Inc, Florida.

Hazarika B N. 2003.Acclimatization of tissue-cultured plants. *Current Science* **85**: 12–25.

Krishna H, Singh S K andPatel V B. 2006.Screening of arbuscular-mycorrhizal fungi for enhanced growth and survival of micropropagated grape (*Vitis vinifera*) plantlets. *Indian Journal of Agricultural Sciences* **76**(5): 297–301.

Malik C P and Singh M B. 1980. *Plant Enzymology and Histo-Enzymology*, p 286. Kalyani Publishers, New Delhi.

Mathur N, and Vyas A. 1995. Influence of VA mycorrhizae on net photosynthesis and transpiration on *Ziziphus mauritiana*. *Journal of Plant Physio*logy **147**: 328–30.

Mathur N and Vyas A. 1999. Improved biomass production, nutrient uptake and establishment of *in vitro* raised *Ziziphus mauritiana* by VA mycorrhiza. *Journal of Plant Physiology* **155** (1): 129–32.

Mondal G, Dureja P and Sen B. 2000. Fungal metabolites from Aspergillusniger AN27 related to plant growth promotion. *Indian Journal of Experimental Biology* **38**(1): 84–7.

Phillips J M and Hayman D S. 1970.Improved procedure for clearing and staining parasitic and vesicular arbuscular mycorrhizal fungi for rapid assessment for infection. *Transactions* of the *British Mycological Society* **55**: 158–61.

Reid C P P. 1979. *Mycorrhiza and Water Stress: The Soil–root Interface*. Academic Press, New York.

Rupnawar B S and Navale A M. 2000. Effect of VA-mycorrhizal inoculation on growth *of* pomegranate layers. *Journal of Maharashtra Agricultural University* **25**(1): 44–6.

Safir G R, Boyer J S and Gerdman J W. 1972. Nutrient status and mycorrhizal enhancement of water transport in soybean. *Journal of Plant Physiology* **43**: 700–3.

Schubert A, Mazitelli M, Ariusso O and Eynard I. 1990. Effect of vesicular-arbuscular mycorrhizal fungi and micropropagated

grapevines: influence of endophyte strain, P fertilization and growth medium. *Vitis* **29**: 5–13.

Sen B. 2000. Biological control: A success story. *Indian Phytopath*ology **53**(3): 243–9.

Singh N V, Singh S K, Singh A K, Meshram D T, Suroshe S S and Mishra D C. 2012. Arbuscular mycorrhizal fungi (AMF) induced hardening of micropropagated pomegranate (*Punica granatum* L.) plantlets. *Scientia Horticulturae* **136**: 122–7.

Singh N V, Chandra R, Meshram D T, Maity A and Pal R K. 2013. Pomegranate: planting material for better quality. *Indian Horticulture* **59**(3): 3–6.

Weatherley P E. 1950. Studies in water relation of cotton plant I. Field measurement of water deficits in leaves. *New Physiologist* **49**: 81–97.

Yang Y, Tang M, Sulpice R, Chen H, Tian S and Ban Y. 2014. Arbuscular mycorrhizal fungi alter fractal dimension characteristics of *Robinia pseudoacacia* L. seedlings through regulating plant growth, leaf water status, photosynthesis, and nutrient concentration under drought stress. *Journal of Plant Growth Regulation* **33**: 612–25.