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Role of Different Nutrient Elements and AgNPs for *In Vitro* Shoot Proliferation of GF-677 Rootstock

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An efficient protocol is described for *in vitro* shoot proliferation from apical shoot of economically important rootstock GF-677 using varied media amalgamations, i.e., ½ Murashige and Skoog (MS), ¾ MS and Woody Plant Medium (WPM) and different concentrations of Silver Nanoparticles (AgNPs) (5, 10, 15, 20, 25 mg L^{-1}). The potential of various concentrations of AgNPs on control of bacterial contamination of GF-677 cultures was also determined. Among different media screened for shoot proliferation of GF-677 rootstock, WPM showed best interaction for shoot number (4.5 shoots/explant) and leaf area (0.52 cm^2) with 20 mg L^{-1} and 25 mg L^{-1} AgNPs, respectively. However, ¾ MS proved to be prosperous media for shoot length (4.92 cm) with 20 mg L^{-1} AgNPs and fresh (755.1 mg) and dry (84.5 mg) weight on 25 mg L^{-1} AgNPs. Multiple shoots with controlled bacterial contamination were also achieved as a result of direct application of AgNPs to the culture media as compared to media devoid of AgNPs. Hence, inclusion of the appropriate AgNPs concentration in the culture medium could provide multiple functions so as to control bacterial contamination in the culture process, resulting in improvement in *in vitro* growth efficiency of GF-677 rootstock.

Key Words: AgNPs, bacterial contamination, GF-677 rootstock, MS, Woody Plant Medium, shoot proliferation

INTRODUCTION

GF-677 (*P. persica* × *P. amygdalus*) is an important rootstock widely used for peach and almond cultivars globally. It is tolerant to drought, iron chlorosis, calcareous and alkaline soils (Ahmad et al. 2003; Sepahvand et al. 2012) and fairly resistant to Plum Pox virus (PPV) (Elektra et al. 2013). Production of this rootstock via hardwoods, semi-hardwoods or soft cuttings is hindered because of very low rooting percentage (Ahmad et al. 2003). Hence, *in vitro* propagation provides an alternative tool to produce true to type plants of economic significance with high multiplication rate, eliminating the difficulties hampered by traditional propagation methods (Elsheik and Khalafalla 2010; Victorio et al. 2012).

Success of *in vitro* grown genotypes largely depend upon optimized nutrient composition of the culture medium (Goncalves et al. 2005), as mineral nutrients play a dynamic role in shoot proliferation of numerous plant species (Aranda-Peres et al. 2009; Kassim et al. 2010). Mineral elements such as AgNPs (Silver nanoparticles) are well known for multiple shoot induction in many plant species through inhibition of ethylene action and for regulating the production of ethylene in cultured vessels, leading to improved growth of plants *in vitro* (Kumar et al. 2009; Sarmast et al. 2011). It is well reported in *Coffea robusta* (Giridhar et al. 2003), *Prunus armeniaca* (Petri et al. 2005), *Sinningia speciosa* (Park et al. 2012), *Helicteres isora* (Chawla and Bansal 2014) and *Malaxis acuminate* (Meena et al. 2010). Moreover, AgNPs are well documented for their antimicrobial activity, more water solubility, less phytotoxicity and low cost availability (Prabhu and Poulose 2012; Mahna et al. 2013), making them a most suitable choice for safer control of contamination rate (Sarmast et al. 2011; Nomiya et al. 2004; Safavi et al. 2011).

In vitro plantlets of GF-677 rootstock were established by determining the role of different carbon sources and growth regulators for shoot multiplication (Ahmad et al. 2003; Ahmad et al. 2007; Younas et al. 2008; Hasan et al. 2010). Considering the novel applications of AgNPs for plant growth improvement as well as contamination control, the role of different levels of AgNPs and varied media compositions was explored, in this study, for *in vitro* shoot proliferation of GF-677 rootstock. More explicitly, the impact of AgNPs for the control of the bacterial contaminants was also determined from *in vitro* cultures.

MATERIALS AND METHODS

Shoot tips of 1–2 cm long were collected from healthy and disease-free peach rootstock "GF-677" from the Horticultural Research Institute (HRI), NARC, Islamabad during the spring season and were rinsed under running tap water for half an hour. Explants were surface-sterilized in 10% (v/v) sodium hypochlorite (NaOCl) solution containing two drops of Tween-20 and thoroughly agitated for 10 min before being rinsed thrice with autoclaved distilled water for 5 min each. Explants were resized to 1.0 cm and cultured on modified MS medium (Murashige and Skoog 1962) consisting of MS macro and micro elements, MS vitamins, 100 mg L^{-1} myo-inositol, 2 mg L^{-1} glycine, 30 g L^{-1} sucrose and 7.0 g L^{-1} agar. After 8 wk of establishment of cultures, varied types of nutrient composition were evaluated for shoot proliferation viz., MS medium reduced to half-strength (½ MS) and three quarters (¾ MS) of their original concentration and Woody Plant Medium (WPM) (Lloyd and McCown 1980) (Tables 1 and 2). Each medium was separately fortified with 2 mg L^{-1} BAP, 0.75 mg L^{-1} IAA and 0.5 mg L^{-1} GA_3 and with different concentrations of AgNPs (Nanocid L-2000; average size 16.6 nm) *viz*., 0, 5, 10, 15, 20, 25 mg L^{-1}. All media containing different concentrations of AgNPs were adjusted to a pH of 5.78 ± 0.02 before autoclaving at 121 °C and 15 psi for 17 min. Cultured explants in 10 mL media were incubated under irradiance of 2,000 lux for 16/8h using white fluorescent tubes (Philips TL 40W/54) at 25 ± 1 °C. Individual shoots were scored for shoot length (cm), shoot number, leaf number, leaf area (cm^2), bacterial contamination (%), shoot dry and fresh weight (g) after 6 wk of incubation. Leaf area (cm^2) was determined by using Leaf Area Meter (LI-3000C) and plantlets were dried at 80 °C for 4 d to determine their dry weights. Each treatment was replicated four times (4 explants/replicates) and means of treatment were separated according to Least Significance Difference *(LSD)* test at $p<0.05$ using Statistix 8.1 software (Steel et al. 1997).

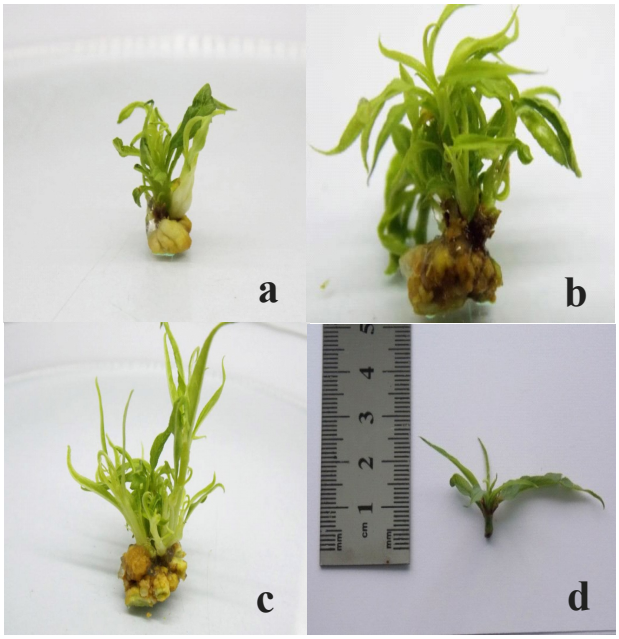

Fig. 1. Response of GF-677 rootstock cultured for 6 wk *in vitro*, exhibiting shoot number on (a) ½ MS + 20 mg L^{-1} AgNPs, (b) ¾ MS + 20 mg L^{-1} AgNPs, (c) WPM + 20 mg L^{-1} AgNPs, (d) Stunted growth of explant on AgNPs free media.

RESULTS

Different compositions of nutrient media, AgNPs and their interactions had remarkable effect on shoot number, shoot length, leaf number, leaf area, shoot fresh and dry weights at $p<0.05$ (Fig. 2). All media compositions (½ MS, ¾ MS and WPM) interacted significantly with 20 mg L^{-1} AgNPs, resulting in 3.0, 4.25 and 4.50 number of shoots/ explant, respectively (Fig. 1. a, b, c; Fig 2a). Significant interaction between media formulations and 20 mg L^{-1} AgNPs was also observed for shoot length with 4 cm, 4.92 cm and 3.8 cm on ½ MS, ¾ MS and WPM subsequently (Fig. 2b). Mean leaf area on ½ MS, ¾ MS and WPM was relatively higher, i.e., 0.45 cm^2, 0.30 cm^2 and 0.52 cm^2, respectively, with 25 mg L^{-1} AgNPs as compared to media compositions without AgNPs (Fig. 2c). For mean leaf number, ¾ MS and WPM were at par with each other with 25 mg L^{-1} AgNPs exhibiting 52.7 leaf number (Fig. 2d). The regenerates obtained at AgNP's free media were stunted and had less number of leaves (Fig. 2d). A significant interaction was also found between media amalgamations and the AgNP concentrations regarding fresh and dry weight of shoots produced in GF-677 rootstock (Fig. 2e & f). Mean fresh weight of explants increased on ½ MS and ¾ MS included with 25 mg L^{-1} AgNPs, except for WPM showing less weight (309.7 mg) on similar concentration of AgNPs. Among different

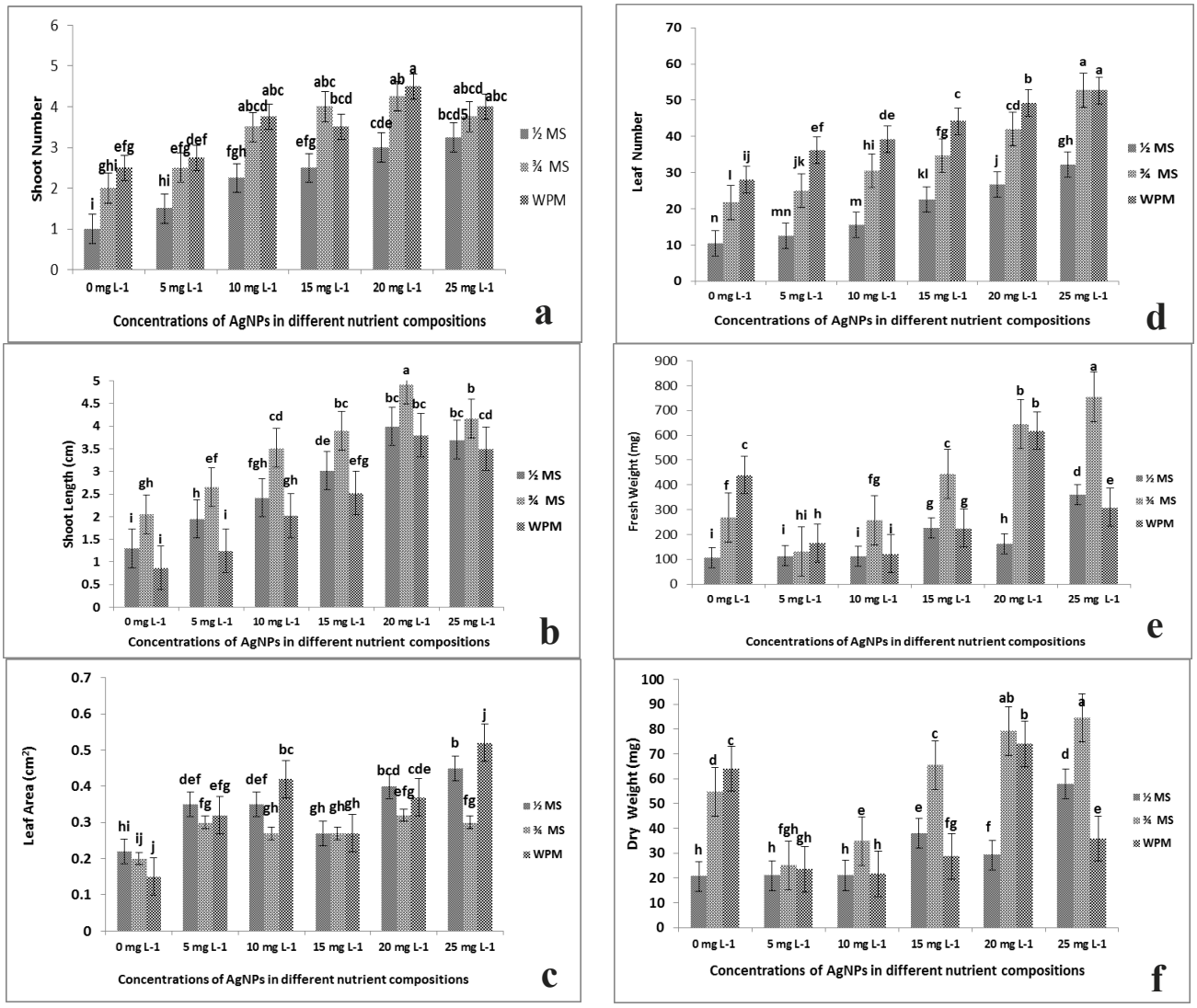

Fig. 2. Interaction of different media compositions with AgNPs concentration for GF-677 rootstock (a) Shoot number, (b) Shoot length (cm), (c) Leaf area (cm^2), (d) Leaf number, (e) Fresh and (f) dry weight (mg). Bars denote the SE.

media screened, WPM resulted in more shoot number and maximum leaf area while ¾ MS performed better for shoot length, shoot fresh and dry weight. Thus, shoot development and elongation varied significantly with different nutrient media as well as with the concentration of AgNPs.

In the present study, bacterial contaminantion was controlled as a result of direct application of AgNPs to the culture media, without deleterious effect on shoot regeneration. Bacterial contamination was controlled in plantlets on medium with 25 mg L^{-1} AgNPs as compared to AgNP-free media (Fig. 3). A linear decline is shown in contamination rate with increase in AgNPs concentration on different media compositions which revealed that addition of AgNPs to the culture media considerably

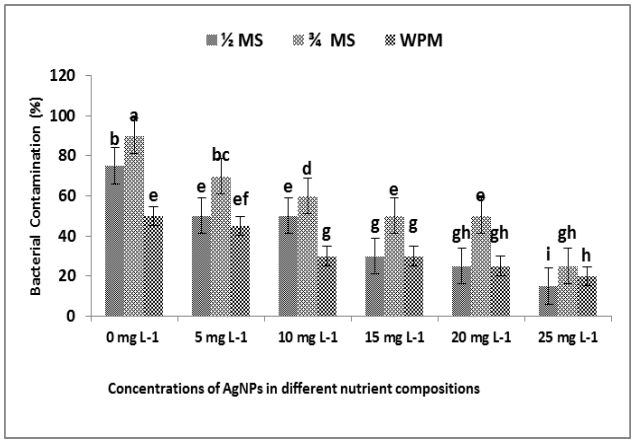

Fig. 3. Influence of various concentrations of AgNPs in different culture media on control of bacterial contamination (%) of GF-677 plantlets.

Table 1. Media composition for *in vitro* shoot proliferation of GF-677 rootstock based on Murashige and Skoog (MS) and Woody Plant Medium (WPM).

	Culture Media (mg L^{-1})		
Mineral Salts	**½MS**	**¾MS**	**WPM**
KNO_3	950	1425	—-
NH_4NO_3	925	1237.5	400
$Ca(NO_3)_2.4H_2O$	—-	—-	556
$CaCl_2.2H_2O$	220	330	96
$MgSO_4.7H_2O$	185	277.5	370
KH_2PO_4	85	127.5	170
K_2SO_4	—-	—-	990
KI	0.415	0.622	—-
$FeSO_4 7H_2O$	13.92	20.85	27.8
Na_2EDTA	18.65	27.97	37.3
$MnSO_4.4H_2O$	11.15	16.72	22.3
H_3BO_3	3.1	4.65	6.0
$ZnSO_4.7 H_2O$	4.3	6.45	8.6
$Na_2MoO_4.2H_2O$	0.125	0.187	0.25
$CuSO_4.5H_2O$	0.0125	0.0187	0.25
$CoCl_2.6H_2O$	0.0125	0.0187	—-

reduces bacterial contamination, with high survival percentage of GF-677 rootstock.

DISCUSSION

Inorganic and organic nutrients are major constitutes of the culture medium used for raising cultures *in vitro* (Ibanez et al. 2003; Akbas et al. 2009; Reddy et al. 2012). Specific nutrient compositions are being designed for *in vitro* multiplication of many fruit trees (Monteiro et al. 2000; Nas and Read 2004; Sotiropoulos et al. 2006; Sotiropoulos 2007). Statistical analysis of plantlets grown on ½ MS, suggested the need for more adjustment of some nutrients in the culture medium for multiple shoot induction. Plantlets regenerated on full-strength WPM had significantly more shoot number, leaf number and leaf area than all other media scrutinized. This could be attributed to high calcium (Ca) contents in full strength WPM because Ca has a main contribution in cell division, enlargement and cell signaling (Reddy 2001; George et al. 2008). Calcium also maintains the integrity of the plasma lemma leading to nutrient retention in cells and regulating the selectivity of ion uptake (Hirschi 2004). The increase in leaf number on full-strength WPM could also be accompanied by the presence of high magnesium (Mg) level. Magnesium plays significant role in cell elongation by activating several enzymes involved in transfer of phosphates whereas, these phosphates are involved in the initiation of shoots which ultimately increases the leaf number (Shaul 2002). Our results are in parallel with Rahman et al. (2014) and Thakur and Kanwar (2008) who attained maximum shoot number per explant in *Pyrus pashia* and *Pyrus pyrifolia* on WPM.

Plantlets cultured on ¾ MS were long and had fresh and dry weight more than the other media screened. The difference of ¾ MS from ½ MS and full-strength WPM is in the levels of macronutrients, i.e., N and K. Thus, it is very feasible to assume that the influence of ¾ MS on biomass accumulation can be characterized by an additional supplement of these macronutrients. Potassium stimulates long distance nutrient flow in cell extension and helps in stomatal movements (George et al. 2008) while, nitrogen is an important constituent of many biological molecules playing a major role in plant growth and development and is readily available to the plants in the form of NH_4^+ and NO_3^- ions (Krouk et al. 2011). Unek et al. 2011 reported that the increase in K concentration enhanced N metabolism and carbohydrate activity which ultimately increased the biomass level. In chrysanthemum the biomass accumulation in terms of fresh and dry weight was found to depend on NH_4^+ and NO_3^- concentration (Sivakumar et al. 2005). Low shoot fresh and dry weight with short length on full-strength WPM might be due to high level of phosphorus (P) in WPM as compared to ½ MS and ¾ MS. Phosphorus is the crucial element for plant growth, photosynthesis, nutrient movement, transformation of sugar into starch and energy transfer, however; more P contents affect the uptake of many essential ions inhibiting the growth *in vitro* (George et al. 2008). Similar observations were carried out by Ciccoti et al. (2008) and Yari et al. (2014) who obtained better results on MS medium in *Malus* spp. and walnut than other media scrutinized.

Novel applications of nano-particles in *in vitro* propagation are emerging increasingly. Role of AgNPs has been explored for multiple shoot induction in many horticultural crops (Turhan 2004; Patil et al. 2011; Pratheesh and Kumar 2012) with inhibition of microbial activity in culture medium (Abdi et al. 2008; Jo et al. 2009). In this study, inclusion of AgNPs in the culture media had remarkable impact on shoot proliferation of

Table 2. Comparison of mineral elements in ½ MS, ¾ MS and WPM used for *in vitro* shoot proliferation of GF-677 rootstock.

Mineral Elements (mg L^{-1})	½MS	¾MS	WPM
N	421.96	632.94	205.966
P	19.37	29.06	38.75
K	391.25	586.87	492.94
Ca	68.75	103.125	124.23
Mg	18.04	27.06	36.09
S	27.74	41.61	236.80
I	0.3175	0.476	-
Cl	120.315	180.47	45.984
Fe	2.79	4.185	5.58
Na	2.515	3.77	5.08
Mn	2.75	4.125	5.485
B	0.55	0.825	1.1
Zn	0.975	1.4625	1.94
Mo	0.0495	0.0742	0.099
Cu	3.18	4.77	6.36
Co	3.11	4.665	-

MS – Murashige and Skoog, WPM – Woody Plant Medium

GF-677 rootstock. With increase in concentration of AgNPs, shoot number, shoot length, leaf number, mean leaf area, shoot fresh and dry weight were influenced significantly. Our results find support from the outcomes of Fuentes et al. (2000) who obtained improved shoot growth in *Coffea canephora* with the addition of AgNPs in culture medium. The stimulatory effect of AgNPs on shoot induction of GF-677 might be due to the interference of silver ion (Ag+) with ethylene signaling (Sarmast et al. 2015). AgNPs are involved in inhibition of ethylene regulation by reducing the receptor capacity to bind ethylene thus, generating ethylene insensitivity in plants and improving plant growth (Zhao et al. 2002). It was also noticed that the AgNO3 can inhibit the leaf and shoot tip abscission of the developed shoots of *Holostemma ada-kodien* Schult. by reducing the inhibitory effect of ethylene (Martin 2002). Plantlets regenerated on nutrient media supplemented with AgNPs were healthier than control and had more growth and shoot number in *Tecomella undulate*, Naga Chilli, *Sinningia speciosa*, *Vitex negundo*, *C. Arabica* and *C. canephora* (Giridhar et al. 2003; Steephen et al. 2010; Aghdaei et al. 2012; Park et al. 2012; Bora et al. 2014).

The antimicrobial effects of AgNPs are well known for controlling the exogenous and endogenous contaminants of woody plants *in vitro* (Anvari et al. 2012). The incorporation of AgNPs in the culture medium showed positive impact toward the control of bacterial contamination in GF-677 rootstock cultures. Our results are in concurrence with the earlier investigators who reported that AgNPs help in removal of bacterial contamination without deleterious effect on plant growth (Panyala et al. 2008; Safavi et al. 2011). Lowest bacterial contamination percentage was found in peach rootstock "GxN15" at 200 mg L^{-1} AgNPs (Arab et al. 2014). Another report by previous examiner showed successful disinfection of *Araucaria excels* stem explants by using AgNPs in culture medium (Sarmast et al. 2011). Dibrov et al. (2002) reported that the chemosmotic activity of Ag^+ may be responsible in affecting the microorganism. Ag^+ ions interact with phospholipids and may substitute the sulphur in the –SH groups of plasma membrane of microorganism to destroy them. Additionally, another study indicated that the damaging effects of Ag^+ are linked with the production of active Ag^+ containing organic compounds and these compounds may attract the microbes and destroy their structure (Tang et al. 2007). AgNP-free media resulted in higher contamination rate on all media compositions, i.e., ½ MS, ¾ MS and full strength WPM. These findings are in accordance with those of Mahna et al. (2013) who achieved maximum contamination rate in *Arabidopsis* seeds and tomato cotyledons on control treatment.

CONCLUSION

The present work brought into focus an effective *in vitro* regeneration protocol of GF-677 rootstock by making adjustments to the mineral composition of the culture medium using apical shoots as explant. The outcomes of this study indicated that nutrient elements have a specific role in *in vitro* shoot regeneration of GF-677 rootstock. It may also be suggested that the addition of the appropriate AgNPs concentration in the culture medium could provide an operative method to control bacterial contamination in the culture process, thus improving the *in vitro* efficiency of GF-677 rootstock.

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