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Research Article

IN VITRO OPTIMIZATION OF THE PROTOCOL FOR MICROPROPAGATION OF PLUM *(PRUNUS SALICINA* L. VAR. METHLEY) FROM NODAL EXPLANTS

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ABSTRACT

The objective of this study was to optimize the protocol for clonal propagation of plum (*Prunus salicina* L. var. Methley) *in vitro*. The nodal explants were cultured on Murashige and Skoog (MS) media supplemented with 6-benzyl amino purine (BAP) or kinetin (KIN) (0.5 - 3 mg/L) alone or in combination with 0.1 mg/L of IBA. The 30 day old *in vitro* derived plantlets were subcultured on MS media supplemented with BAP alone or in combination with 0.1 mg/L IBA. For rooting half strength MS media supplemented with indole-3-butyric acid (IBA) or indole -3- acetic acid (IAA) (0.5 - 3 mg/L) were used. The highest shoot induction response (92 %) was observed on MS media supplemented with 0.5 mg/L BAP and 0.1 mg/L IBA with an average number of 2.08±0.14 shoots per explants and 2.3±0.26 cm average shoot length. The optimum rooting response (100 %) was observed on 1.0 mg/L IBA with an average number of 4.34±1.15 roots per shoot and 3.57±0.08 cm average root length. Therefore, these concentrations are recommended for *in–vitro* micropropagation of P*runus salicina* L. var. Methley.

Key words: Plum, Nodal explants, Micropropagation, Plant growth regulators, MS media

INTRODUCTION

Breeding of temperate fruit tree species via conventional method is a difficult and expensive process due to heterozygosity, polyploidy, long breeding cycles, and lengthy field trial procedures (Canli and Tian, 2008). Most temperate fruit species are self-sterile and extremely heterozygous and most scion and root stocks cultivars don't come true-to-type if propagated using their own, seldom fertile, seeds. Clonal propagation is the most important method of commercial production of the majority of horticultural crops throughout the world (Davies *et al*., 1994). Thus, it is possible to produce plants in large numbers in short time from single individual

(Razdan, 1993). Tissue culture has numerous potential applications for highland fruits including propagation of rootstocks, own-rooted scion cultivars, virus-free stock plants, and elite genotypes (Hutchinson and Zimmerman, 1989).

Plums are temperate fruit trees classified under the genus *Prunus and* family *Rosecea.* They are produced and cultivated globally for its edible fruit. This fruit is rich in carbohydrates, proteins, fat, vitamins and minerals (Bofung *et al*. 2002, Folta, 2009). The plum fruit can be used as a staple food to consume fresh, juice, dried food or pruned food, oil, ingredients to produce dyes, jellies and jams (Sonwa *et al*., 2002). The plum fruit crop is produced all over the world. According to FAOSTAT report (2012) studied from 2007 to 2012 showed that about 50% of the total plum fruit production is covered by five countries: China, USA, Brazil, India and European Union. Among these countries, China is the leading producer and exporter of plum for global market (FAOSTAT, 2012). This report also indicated that the global share of plum fruit production in Africa is about 2.8 % (150 thousand tones) in which most of its production is covered by South Africa.

Plum is typically propagated through seeds and vegetatively by cuttings and grafting (Anegbeh *et al.*, 2005 and Zou, 2010). Although propagation by cutting is preferred it is season dependent, laborious and requires large area for propagation. The problems exhibited for plum propagation by seed, stem cuttings and grafting can be overcome by in-*vitro* culture or micro-propagation. The micro-propagation is performed by taking stem node, shoot tip, leaf, root and seed explants (Minocha *et al*., 2000). Tantos *et al* (2001) showed that micropropagation of plum from shoot tip cultures in MS media supplemented with 0.5 - 2 mg/L of 6-benzyl amino purine (BAP) with only few or no auxin. Some *prunus* cultivars respond better to lower concentrations of BAP from axillary buds (George, 1993). The most widely known plum fruit types in Ethiopia are Pioneer and Methley varieties. These plum varieties are high yielding and adapted to altitudes greater than 2300 meters above sea level. Conventionally these plum varieties are propagated by grafting of these scion varieties with peach root stock (Rothweiler-Sporn, 2012). Micropropagation offers a suitable method to provide the fruit plant growers sufficient, true to type, disease- free planting materials. So far, there is no a protocol developed for micropropagation of Methley plum variety elsewhere in the world and Ethiopia. Furthermore, the protocol developed for one plant variety couldn't work for the others as *in-vitro* response is genotype dependent. Therefore, the objective of the present study was to optimize the protocol for micropropagation of Plums (*Prunus salcina* L. var. Methley).

MATERIALS AND METHODS

Plant materials and Preparation

The mother plant materials for this study were obtained from four year old field-grown plum trees (*Prunus salicina* L. var. Methley) at Shenkor Mesk, temperate fruit plant nursery site, North Gondar, Ethiopia. The experimental activities were carried out in the plant tissue culture laboratory, Department of Biotechnology in

University of Gondar, Ethiopia from February 2015 to October 2015. The explants were prepared by taking growing nodal segment of the plants by removing extra leaf sheath. Explants were washed thoroughly in soap solution for 5 minutes and under running tap water for 20 minutes to remove soil and other superficial contamination. Then the nodal segments were rinsed 3 times in double distilled water and kept for 20 minutes in cold anti-oxidant solution (150 mg/L citric acid and 100 mg/L ascorbic acid) to avoid browning problem of the tissue in the culture. Surface sterilization of these explants were done by treatment with 70% (V/V) alcohol for 30 seconds, 0.1 % (W/V) mercuric chloride ($HgCl_2$) for 7 minutes, 2% (W/V) sodium hypochlorite (NaOCl) for 20 minutes with a few drops of Tween-20 solution with subsequent rinsing with double distilled water. Finally, the explants were thoroughly rinsed 5 times with sterile double distilled water to remove all traces of disinfectants. All the steps of the sterilization process had been done under aseptic conditions in laminar air flow hood. Then these plants were kept in sterile double distilled water, trimmed to 2 cm long under aseptic conditions and cultured on the already prepared Murashige and Skoog (MS) medium (1962) supplemented with different concentrations of plant growth regulators (PGRs). The pH of the MS media was adjusted to 5.8 before adding 0.8% (W/V) of the agar. Then it was taken for autoclaving at 120^{o}C and 104 kpa for 15 minutes.

Shoot initiation and multiplication

Full strength MS media supplemented with five levels of 6-benzyl amino purine (BAP) or kinetin (KIN) with concentrations (0.5, 1, 1.5, 2 and 3 mg/L) alone or in combination with 0.1 mg/L of IBA were used for shoot initiation. Shoot initiations on MS media supplemented with cytokinins (BAP or KIN) alone or in combination with 0.1 mg/L of IBA were compared for better response. Depending on culture initiation using these PGRs, the MS media supplemented with BAP alone or in combination with 0.1 mg/L IBA was selected for shoot multiplication. The basal medium (free of PGRs) was used as a control for this study. After the inoculation of the explants, the culture jars were kept at dark for three days. Then the culture jars were maintained at 25 ± 2 ^{o}C in 16 hour photoperiod and 2700 lux light intensity under cool white fluorescent tubes. The data were recorded for the percent of culture response, average number of shoots per explants, average length of shoots (cm) after four weeks.

Effect of auxin type and concentrations on root growth

Well developed and elongated shoots from the multiplication media were excised at V- shape and transferred to half strength MS media supplemented with IBA or indole -3- acetic acid (IAA) at different concentrations (0.5, 1, 1.5, 2, and 3 mg/L). Half strength MS basal medium (free of PGRs) was used as control. The plant culture growth room chambers had the same culture conditions (temperature, photoperiod and light intensity) with shoot initiation study. Then the cultures were kept in dark conditions for the first three days to promote root initiation. After four weeks on rooting

medium: rooting percentage, mean number of roots/ plantlet and average root length (cm) were recorded.

Experimental Design and Statistical data Analysis

All the experimental activities were laid out in completely randomized design (CRD) in three replications and each treatment consists of 12 explants. Statistical data analysis was done by Statistical Package for Social Sciences (SPSS) version 16.0 software. Analysis of variance (ANOVA) was done for the comparison of treatments and Duncan's multiple range test were used to detect the significant difference among treatment means at $p \leq 0.05$.

RESULTS

Shoot initiation

The effect of different concentrations of BAP or KIN alone or in combinations with 0.1 mg/L of IBA on the culture initiation of the nodal segments of plum (P*runus salicina* L. var. Methley) was investigated. The percentage of response of the explants *in vitro* for different concentrations of these PGRs is indicated on Table 1 and Table 2. There was significant difference for shoot number (0.0 - 2.08±0.14) and shoot length (0.0 – 2.33 ± 0.29 cm) on MS media supplemented with BAP alone or in combination with 0.1 mg/L IBA after four weeks as indicated on Table 1 and Fig. 1. The maximum shoot regeneration response of the explants were 91.67% on MS media supplemented with 0.5 mg/L BAP in combination with 0.1 mg/L IBA. In this media combination an average number of shoots per explants and shoot length were 2.08±0.14 and 2.3±0.26 cm, respectively. There was also significant difference for shoot number (0.0 - 1.33±0.29) and shoot length (0.0- 1.83±0.29 cm) on MS media supplemented with BAP alone or in combination with 0.1 mg/L IBA after four weeks as indicated on Table 2. The shoot regeneration response of KIN alone and in combination with 0.1 mg/L IBA in all treatments were poor compared with BAP treatments after four weeks as indicated on Table 1 and 2. There was no response for MS basal medium for shoot initiation of P*runus salicina* L. var. Methley.

Table 1. Effect of different concentrations of BAP alone or in combination with 0.1mg/L IBA on shoot initiation from the nodal explants of *P. salcina* L. var. Methley.

Plant growth regulators (mg/L)		Percentage of response	Average number of shoots per explant	Average shoot length (cm)
BAP	IBA			
Control	0.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
0.5	0.0	86.11 ± 4.82^{ef}	1.93 ± 0.2^{ef}	1.90 ± 0.10^{fg}
1.0	0.0	80.56 ± 9.62^{def}	1.78 ± 0.31^{e}	1.72 ± 0.25^{ef}
1.5	0.0	72.22 ± 4.81^{cd}	1.07 ± 0.06^{cd}	1.33 ± 0.57^{de}
2 .0	0.0	66.56 ± 8.50^{c}	0.80 ± 0.00^{bc}	1.13 ± 0.33^{cd}
3.0	0.0	47.11 ± 9.43^{b}	0.55 ± 0.13^{b}	0.56 ± 0.05^{b}

0.5	0.1	91.67±8.34^{f}	2.08±0.14^{f}	2.3±0.26^{g}
1.0	0.1	88.89±4.81^{ef}	1.86±0.35^{ef}	2.33±0.29^{g}
1.5	0.1	77.78±4.80^{cde}	1.25±0.00^{d}	1.85±0.05^{fg}
2.0	0.1	72.22±4.81^{cd}	0.94±0.10^{c}	1.63±0.23^{ef}
3.0	0.1	52.67±4.62^{b}	0.64±0.10^{b}	0.84±0.27^{bc}

*The means followed by the same letter in a column are not statistically different according to the Duncan's multiple range test ($P \leq 0.05$).

Table 2. Effect of different concentrations of KIN alone or in combination with 0.1mg/L IBA on shoot initiation from the nodal explants of *P. salcina* L. var. Methley.

Plant growth regulators (mg/L)		Percentage of response	Average number of shoots per explant	Average shoot length (cm)
KIN	IBA			
Control	0.0	0.0 ±0.0^{a}	0.0 ±0.0^{a}	0.0 ±0.0^{a}
0.5	0.0	75.00±0.00^{fg}	1.00±0.00^{d}	1.78±0.29^{e}
1.0	0.0	66.44±14.62^{ef}	0.78±0.03^{cd}	1.27±0.25^{d}
1.5	0.0	63.78±5.01^{def}	0.75±0.00^{cd}	0.91±0.08^{b}
2 .0	0.0	52.67±4.62^{cd}	0.66±0.09^{bc}	0.88±0.11^{b}
3.0	0.0	38.89 ±4.82^{b}	0.45±0.05^{b}	0.79±0.07^{b}
0.5	0.1	83.33±0.00^{g}	1.33±0.29^{e}	1.83±0.29^{e}
1.0	0.1	72.22±9.61^{fg}	0.93±0.12^{d}	1.33±0.29^{d}
1.5	0.1	66.67±0.00^{ef}	0.92±0.14^{d}	0.96±0.06^{bc}
2.0	0.1	58.22±8.34^{de}	0.75±0.25^{cd}	0.95±0.08^{bc}
3.0	0.1	44.45±4.81^{bc}	0.61±0.13^{bc}	0.86±0.11^{b}

*The means followed by the same letter in a column are not statistically different according to the Duncan's multiple range test ($P \leq 0.05$).

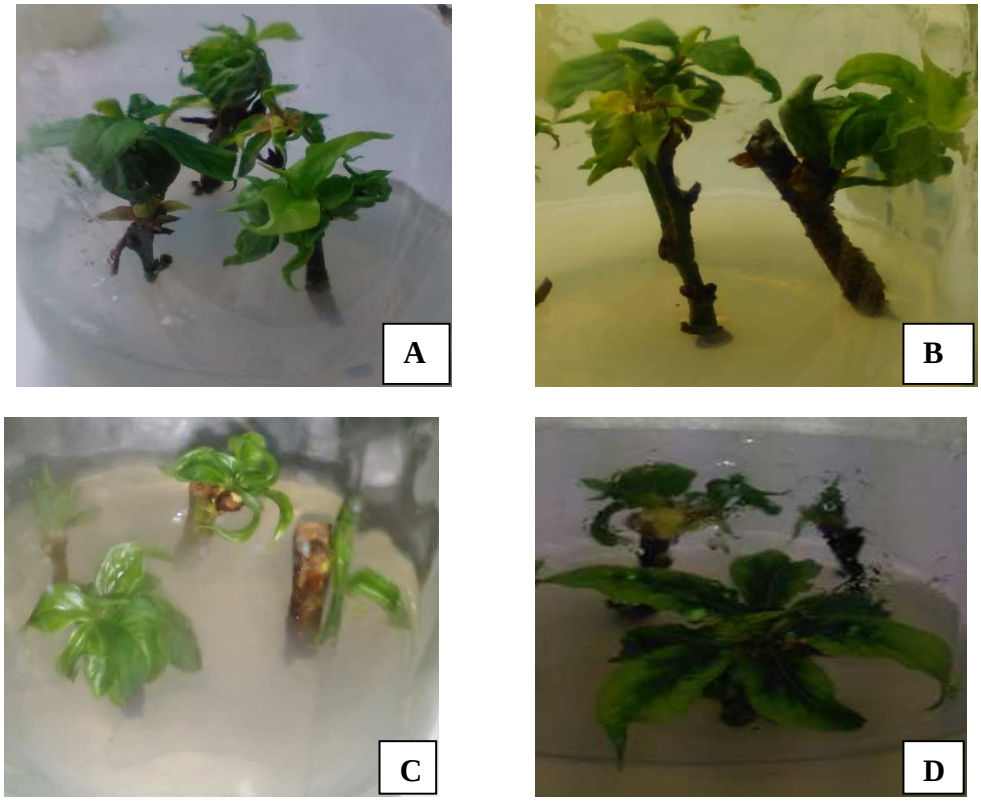

Figure 1: Shoot initiation from nodal explants of plum (P*runus salicina* L. var. Methley) on MS media supplemented with different concentrations of PGRs after four weeks: (A) 0.5 mg/L BAP in combination with 0.1 mg/L IBA, (B) 0.5 mg/L BAP, (C) 1 mg/L BAP and (D) 1 mg/L BAP in combination with 0.1 mg/L IBA.

Shoot Multiplication

The shoot initiation on MS media supplemented with BAP alone or in combination with 0.1 mg/L IBA showed better response compared with KIN alone in combination with 0.1 mg/L IBA. There was significant difference for average number of shoots per explants and average shoot length (cm) for plantlets grown on shoot multiplication MS media supplemented with BAP in combination with 0.1 mg/L IBA. The maximum average number of shoots (3.12±0.10) was observed on MS media supplemented by 0.5 mg/L BAP in combination with 0.1 mg/L IBA whereas maximum average shoot length (2.96±0.06 cm) was obtained on MS media supplemented by 1.0 mg/L BAP in combination with 0.1 mg/L IBA after four weeks as indicated on Table 3 and Fig. 2. There was minimum response for MS basal medium for shoot multiplication.

Table 3. Effects of different concentrations of BAP in combination with 0.1mg/L of IBA on shoot multiplication of *P. salcina* L. var. Methley.

BAP (mg/L)	Average number of shoots per plantlet	Average shoot length (cm)
Control	1.00±0.00[a]	1.53±0.50[a]
0.5	3.12±0.10[e]	2.33±0.23[c]
1.0	2.50±0.50[d]	2.96±0.06[bc]
1.5	2.00±0.00[c]	2.59±0.40[b]
2.0	1.42±0.14[b]	2.45±0.40[b]
3.0	1.00±0.00[a]	0.74±0.24[a]

*The means followed by the same letter in a column are not statistically different according to the Duncan's multiple range test ($P \leq 0.05$).

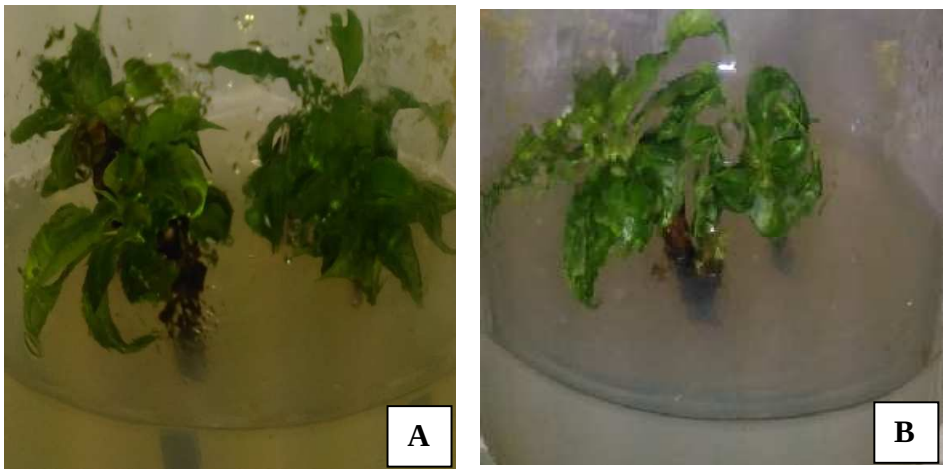

Figure 2: Effects of different concentrations of PGRs for shoot multiplication from shoots taken from MS initiation medium of plum (P*runus salicina* L. var. Methley) after four weeks: (A) 0.5 mg/L BAP in combination with 0.1 mg/L IBA, (B) 1 mg/L BAP in combination with 0.1 mg/L IBA.

Root initiation

The root induction on half strength MS media supplemented with IBA showed significant differences ($P \leq 0.05$) after four weeks as indicated on Table 4 and Fig. 3. The highest percentage of rooting was observed MS medium supplemented with 1.0 mg/L IBA. In this treatment, average number roots and root length were 4.34±1.15 and 3.00±0.08 cm, respectively. It is also observed that increase in the concentrations of IBA had decreased the rooting ability of *P. salcina* L. var. Methley. However, there was no rooting response for all IAA treatments and control ones after four weeks in this study.

Table 4. Effects of different concentrations of IBA for root induction on half strength MS medium in *P. salcina* L. var. Methley.

IBA (mg/L)	Percent response	Average number of roots per plantlet	Average root length (cm)
Control	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
0.5	66.56±0.20[c]	2.78±1.25[c]	2.17±0.29[d]
1.0	100±0.00[d]	4.34±1.15[d]	3.57±0.08[e]
1.5	66.67±0.00[c]	2.28±1.11[c]	2.38±0.35[d]
2.0	33.33±0.00[b]	1.11±0.19[b]	1.98±0.48[c]
3.0	22.22±0.00[b]	0.45±0.39[a]	0.89±0.78[b]

*The means followed by the same letter in a column are not statistically different according to the Duncan's multiple range test ($P \leq 0.05$).

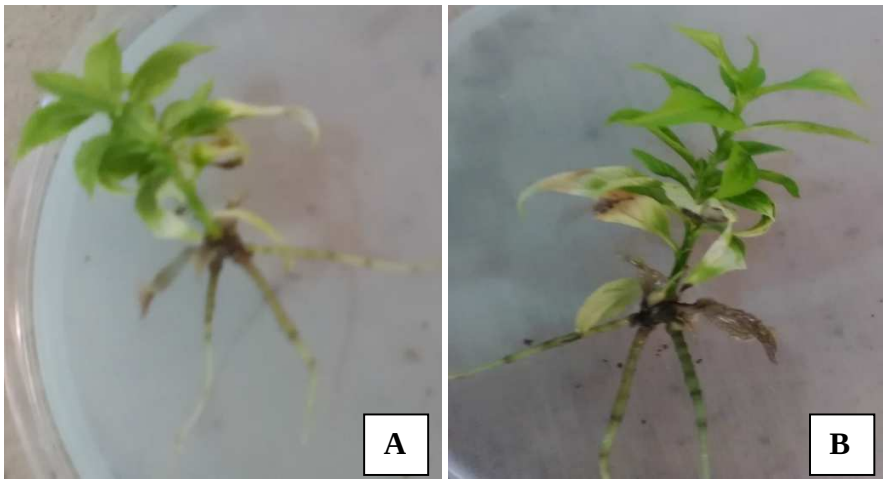

Figure 3: Root initiation of plum (P*runus salicina* L. var. Methley) plantlets on half strength MS media supplemented with different concentrations of IBAs after four weeks: (A) 0.5 mg/L IBA, (B) 1mg/L IBA.

DISCUSSION

In the present study maximum multiple shoots (3.12±0.10 shoots per explant) were observed on MS media supplemented with 0.5 mg/L BAP supplanted with 0.1 mg/L of IBA whereas maximum average shoot length (2.96±0.06 cm) was obtained on MS media supplemented with 1.0 mg/L BAP in combination with 0.1 mg/L IBA after four weeks. BAP plays an important role for shoot elongation and proliferation of the auxiliary shoots. The addition of auxins along with cytokinins increased the responses of the culture, shoot length and shoot number in *Prunus* species (Silva *et al.*, 2003, Mansseri-Lamrioui *et al.*, 2011, Ostadsharif *et al.*, 2014, Ruzic and Vujovic, 2008 and Zou 2010). Therefore MS media supplemented with 0.5 mg/L BAP in

combination with 0.1 mg/L of IBA was the most effective combinations of cytokinin and auxin for shoot induction and shoot multiplication. MS media supplemented with BAP in combination with IBA performed better proliferation of shoots compared with supplementing KIN and IBA in *Prunus* species (Zou, 2010). Similar results were observed in the present study for new shoot formation in BAP concentrations alone or in combination with 0.1mg/l IBA. Whereas KIN alone or in combination with 0.1 mg/L of IBA showed less shoot proliferation capacity compared with BAP. This might be due to the physiological role of BAP to break the apical dominance and enhance growth of new shoots at low concentrations (Pruski *et al.*, 2005, Edriss *et al.*, 2014). Therefore, the results observed in this study are in agreement with the previous reports for week response of shoots for KIN (Silva *et al.*, 2003, Pruski *et al.*, 2005, Tian *et al.*, 2007, Zou, 2010 and Edriss *et al.*, 2014).

Root induction from the shoots of *P. salcina* L. var. Methley on half strength MS medium was preferred to prevent the callusing effects of the full strength MS media on the cut ends of the shoots. Therefore, lower concentrations of minerals in the media are more efficient for root induction *in- vitro* (George and Sherrington, 1984). The superiority of half strength MS medium for root induction had been reported by various researchers (Hossain *et al*., 2003, Zou, 2010, Choudhary *et al.*, 2015 and Alam & Barua, 2015). In the present study the highest percentage of root induction (88.9%), maximum number of roots per explants (4.34±1.15) and maximum root length (3.00±0.08 cm) were observed on half strength MS media supplemented with 1.0 mg/L of IBA. Auxins had crucial role on the rooting of shoots. Previous reports on temperate fruits showed that IBA had enhanced root response (Bandeira *et al.*, 2012, Mansseri-Lamrioui *et al.*, 2011, Vujovic *et al.*, 2012 and Zou, 2010). In the present study the use of IAA for root induction was not effective compared to IBA. During micropropagation, IAA is rapidly photo-oxidized by an enzyme oxidase compared with IBA (Housman, 2003; Yadollahi and NazaryMoghadam, 2012 and Mansseri-Lamrioui *et al.*, 2011). Therefore, the slow movement and delayed degradation of IBA may be the primary reason for better performance of root response compared with IAA. Thus, the weak performance of IAA may be related to photochemical and/or enzymatic oxidation. *In vitro* rooting can be enhanced by keeping cultures in the dark for short period of time prior to transferring to light condition (Housman, 1993; Yadollahi and Moghadam, 2012 and Mansseri-Lamrioui *et al.*, 2011).

CONCLUSION

In the present study the micropropagation protocol was developed for plum (P*runus salicina* L. var. Methley) using nodal segments. The induction and proliferation of shoots and roots of these plants was dependent on plant growth regulators. The number of newly formed shoots varied with concentration of different plant growth regulators. The best shoot response and proliferation was obtained on full strength MS media supplemented with 0.5 mg/L BAP in combination with 0.1 mg/L of IBA. Whereas the best rooting

response was observed on half strength MS media supplemented with 1.0 mg/L IBA. Therefore, these concentrations are recommended for *in–vitro* micropropagation of sufficient, true to type and disease free plants of P*runus salicina* L. var. Methley.

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