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Conservation of Endangered Hassawi Peach (*Prunus persica* L.) Through Micropropagation

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Abstract: The objective of this study was conducted to establish a micropropagation system suitable for Hassawi peach (*Prunus persica* L.), an endangered landrace variety adapted to the hot arid climatic conditions of Al-Hassa, Saudi Arabia. This study examined the effect of some plant growth regulators on the response of node explants. The best shoot multiplication and growth was obtained using MS medium supplemented with 0.5 mg L⁻¹ 6-benzylaminopurine (BA) and 0.1 mg L⁻¹ Indole-3-butyric acid (IBA). This treatment gave the highest shoot multiplication (5.8 shoots per explant), shoot elongation (4.7 cm) and leaf number (19.3 leaves per shoot). During rooting stage, the best rooting was associated with shoots cultured on ½ MS medium containing 0.5 mg L⁻¹ IBA. This treatment gave 92% rooting with maximum root length of 6.8 cm and highest average number of root (5.4 roots per shoot). Plantlets exhibited normal growth and phenotype upon transfer to soil with 95% survival. This micropropagation system could contribute to the conservation and restoration of this threatened peach germplasm.

Key words: *In vitro*, landrace, micropropagation, organogenesis, peach, tissue culture

INTRODUCTION

Peach (*Prunus persica* L.) is a deciduous tree species belonging to the family Rosaceae and can grow up to 4-10 m tall producing edible juicy stone fruit (Martinez-Gomez *et al.*, 2003). The fruits are globally popular for its pleasant aroma and delicious taste in addition to its amenability for industrial food processing (Layne and Bassi, 2008). Peach is one of the most important fruit crops in the world in terms of production which reached 18 million tons with a cultivated area of 1.6 million ha (FAOSTAT, 2010). It is thought that this species has originated in China (Wang, 1985) and currently commercially cultivated in many temperate countries around the world (Akçay and Uzunoç, 2005; Melgoza-Villagomez *et al.*, 2009).

Historically, the Al-Hassa Oasis which is located at the Eastern Province of Saudi Arabia is predominated millions of palm trees which created a microclimate suitable for the adaptation and wide spread of various fruit species including berries, grape, pear, apple and peach. Socioeconomical changes and increasing water deficit during the last five decades have negatively impacted plant genetic diversity in important agricultural regions of Saudi Arabia (Rogers and Lydon, 1994). Many of the fruit species once grown in Al-Hassa Oasis has dwindled to a few scattered trees grown in some small family farms for sentimental value. Real conservation

efforts of these locally adapted genetic resources are almost nonexistent. The current study was set forth in an effort to rescue the threatened population of peach trees in Al-Hassa Oasis, hint the name Hassawi peach.

Conservation of the diversity of genetic resources of global plant ecosystems has become a major international concern. The loss of increasingly large numbers of plant species through habitat destruction threatens the availability of a diverse plant germplasm base needed for crop improvement to enhance world food security (Holden *et al.*, 1993). Conservation efforts are most essential at the current time to prevent the potential loss of germplasm to the unfavorable conditions imposed by the global climate changes (Jain, 2010). Successful conservation and restoration schemes require a sufficient strategy for the maintenance and propagation of the threatened plant materials. Tissue culture methodology proved superior to the traditional propagation and conservation methods, especially for vegetatively propagated plant species since, it provides large quantities of disease-free clonal propagules in a relatively short time (Malavasi and Predieri, 1988). The availability of a micropropagation protocol suitable for Hassawi peach would contribute to preventing the extinction of this landrace germplasm by multiplying the remaining trees. In addition, this protocol provides an excellent means for *in vitro* conservation (Engelmann, 1991; Shibli *et al.*, 2006; Jain, 2011).

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Several studies demonstrated micropropagation of peach through organogenesis (Ruzic *et al.*, 1984; Martinelli, 1985; Morini and Concetti, 1985; Malavasi and Predieri, 1988; El-Deen *et al.*, 1998; Ahmad *et al.*, 2003; Alsalihiy *et al.*, 2004; Cos *et al.*, 2004; Younas *et al.*, 2008; Mahmood *et al.*, 2009). Others have obtained plant regeneration through somatic embryogenesis (Bhansali *et al.*, 1991). As a breeding tool (Anderson *et al.*, 2002) developed a protocol for embryo culture. These studies have developed protocols designed for specific peach cultivars however, none investigated Hassawi peach. Moreover, studies with *Prunus* spp. have demonstrated that hormonal manipulations are necessary to optimize the requirements for specific cultivars. Obvious differences were noted in response to different plant growth regulators including auxins (Alsalihiy *et al.*, 2004; El-Hammady *et al.*, 2005), cytokinins (Teixeira *et al.*, 2004) and gibberellins (Zaied, 1997).

Our goal was to establish a micropropagation system suitable for Hassawi peach using node culture. The specific objectives were to examine:

- The effect of 6-benzylaminopurine (BA) and indole-3-butyric acid (IBA) combinations on shoot initiation and multiplication
- The influence of IBA concentration on root induction of the regenerated shoots
- The status of plant survival following acclimatization and soil transfer

MATERIALS AND METHODS

Explant preparation: This study was carried out at the Department of Agricultural Biotechnology, College of Agricultural and Food Sciences, King Faisal University, Al-Hassa, Saudi Arabia. Branch cuttings (10 cm long) were collected in March/April 2011 from peach trees of Hassawi variety, grown at a local farm at Al-Omran, Al-Hassa, Saudi Arabia. Leaves were removed and the twigs were thoroughly washed in running tap water and disinfected in 70% ethanol for 1 min, followed by 20% commercial bleach (Clorox) containing 3 drops Tween 20 per 100 mL solution for 20 min and then rinsed 3 times with sterilized distilled water to remove traces of the disinfection. Node explants were cut at 2.5 cm long; each node contained a single axillary bud. The nodes were cultured individually in an upright position in culture tubes containing initiation media.

Culture medium and conditions: The culture medium consisted of MS inorganic salts (Murashige and Skoog,

1962) supplemented with 1 mg L⁻¹ thiamine-HCl, 1 mg L⁻¹ pyridoxine-HCl, 1 mg L⁻¹ nicotinic acid, 100 mg L⁻¹ myo-inositol, 30 g L⁻¹ sucrose and 7 g L⁻¹ agar [Agar-agar/Gum agar] (Sigma Chem. Co., St. Louis, MO).

Different growth regulator combinations were added to the culture medium according to the culture stages. For culture initiation and multiplication stages, the explants were cultured on full strength MS medium supplemented with combinations of BA at 0.25, 0.5, 1 and 2 mg L⁻¹ and IBA at 0.025, 0.1 and 0.4 mg L⁻¹. For the rooting stage, regenerated shoots were cultured on a medium consisting of ½ strength MS medium augmented with IBA at 0, 0.25, 0.5 and 1 mg L⁻¹.

All media were adjusted to pH 5.8 and autoclaved at 121°C and 1.1 kg cm⁻² for 15 min. Prior to autoclaving, the media were dispensed in 150×25 mm culture tubes at 15 mL per tube for the initiation and rooting stages and Magenta vessels at 50 mL per vessel for the shoot multiplication stage. All the culture stages were incubated at 24±2°C under a 16 h photoperiod provided by cool-white florescent light (40 µmol m⁻² sec⁻¹).

Shoot initiation and multiplication: The cultures were maintained for a total of 8 week during which they were transferred fresh media once at a 4 week interval. Following the first 4 week of culture, explant survival and initial growth were assessed based on a scoring system described by Pottino (1981) who expresses the responses as:

- 1: Negative
- 2: Below average
- 3: Average
- 4: Good
- 5: Excellent

The regenerated shoot/bud clusters were isolated from the original explant and transferred to a fresh medium containing the corresponding hormonal treatments. After incubation for additional 4 week, shoot multiplication was evaluated based on number of shoots per explant, mean shoot length and number of leaves per shoot.

Rooting and acclimatization: The regenerated shoots, 2 cm in length, were separated and transferred to the rooting medium. After 6 week, data pertaining to rooting percentage, root number and root length were recorded. Plantlets were removed from the culture vessel and washed thoroughly in sterile distilled water to remove agar residues. The roots were impressed in 3 g L⁻¹ Benomyl fungicidal solution for 10 min and planted in 8 cm pots filled with soil mixture consisting of equal volume of peat moss, sand and vermiculite. The potted

plantlets were covered with plastic bags to maintain humidity and incubated in the culture room for 3 week during which the bags were gradually opened to expose the plantlets to ambient conditions. Plants that survived acclimatization were transferred to a greenhouse for further growth.

Statistical analysis: The shoot multiplication experiment was conducted as a randomized factorial design with 2 main factors, IBA concentration at 3 levels and BA concentration at 4 levels with 10 replications. The data were statistically analyzed based on analysis of variance (ANOVA) and the means were separated where appropriate using the Least Significance Difference (LSD) at 5%. The rooting experiment was conducted as a single factor randomized design with the main factor IBA concentration at 4 levels with 15 replications and the data were compared using t-test.

RESULTS AND DISCUSSION

Initial explant responses: Within the first 2 week of culturing, the preexisting axillary buds in the nodal explants opened and began to form shoots in the

following 2 week (Fig. 1a). The results of the general assessment of explants in terms of survival and initial growth made at the end of week 4 are shown in Table 1. Based on total scores, the most responsive treatments, in a descending order, were: 0.5 mg L⁻¹ BA and 0.1 mg L⁻¹ IBA, 1 mg L⁻¹ BA and 0.025 mg L⁻¹ IBA and 0.25 mg L⁻¹ BA and 0.1 mg L⁻¹ IBA with a total score of

Table 1: The interaction effect of BA and IBA on proliferation stage of Hassawi peach after 4 weeks of culture initiation

Treatments (mg L ⁻¹)		Response score*			
BA	IBA	Explant growth	Bud multiplication	Shoot formation	Total score
0.25	0.025	2	1	2	5
	0.1	3	3	4	10
	0.4	4	1	4	9
0.5	0.025	4	1	4	9
	0.1	5	4	5	14
	0.4	4	1	4	9
1.0	0.025	4	4	4	12
	0.1	3	3	3	9
	0.4	3	1	3	7
2.0	0.025	3	1	3	7
	0.1	4	1	4	9
	0.4	4	1	4	9

*Rating of individual parameters: 1: Negative, 2: Below average, 3: Average, 4: Good and 5: Excellent

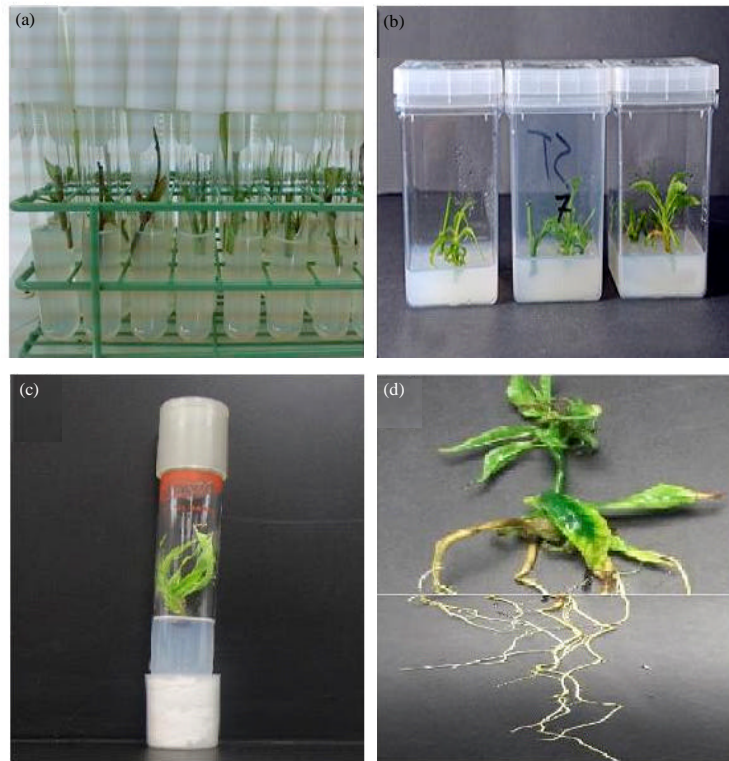


Fig. 1(a-d): Stages of *in vitro* regeneration of Hassawi peach: (a): Culture establishment (b): Bud and shoot proliferation (c): Shoot elongation (d): Root formation

14, 12 and 9 out of a maximum possible total score of 15. The best treatment (0.5 mg L⁻¹ BA and 0.1 mg L⁻¹ IBA) was associated with highest explant growth, shoot formation and shoot multiplication with scores of 5, 4 and 5, respectively. Although, other treatments resulted in a comparable explant growth and shoot formation, they scored low relative to bud multiplication; consequently, the observed low total score.

Shoot multiplication: The resultant shoots grew vigorously and developed without any visible deformities or callus formation (Fig. 1b, c). The analysis of variance of the data collected 8 week after culture initiation indicated that the number of shoots formed per explant was significantly affected by an interaction between BA and IBA concentrations (Table 2). The effect of these growth regulators on shoot multiplication is illustrated in Fig. 2. The best response (5.8 shoots per explant) was obtained using MS medium containing a combination of 0.5 mg L⁻¹ BA and 0.1 mg L⁻¹ IBA. Whereas, the lowest number of shoots per explant was associated with the treatment containing 0.25 mg L⁻¹ BA and 0.025 mg L⁻¹ IBA.

The effectiveness of MS medium for peach micropropagation is collaborated by other investigators (Alsalihi *et al.*, 2004; Borkowska *et al.*, 2008; Younas *et al.*, 2008). In other *Prunus* species such as almond, MS medium was also found to sustain shoot multiplication and growth (Caboni and Damiano, 1994;

Saeed, 1998; Cos *et al.*, 2004; Yang-Ning *et al.*, 2004). However, the best multiplication rate expressed in bud number was obtained using ¾ strength MS medium. In contrast (Morini and Concetti, 1985) found that peach explants cultured on woody plant medium, WPM (Lloyd and McCown, 1981) containing 1.2 mg L⁻¹ of BAP exhibited improved shoot growth and multiplication.

Studying other components of the culture medium, Gurel and Gulsen (1998) found that 50-60 g L⁻¹ sucrose induced the best shoot multiplication and growth. In contrast, the medium used in the current study utilized a lower sucrose concentration (30 g L⁻¹). Reducing the sucrose concentration to the minimum requirement can reduce the relatively high expenses associated with plant tissue culture, an important aspect in commercial micropropagation. Minimizing the agar concentration can also contribute to cost reduction. This was accomplished by Rodrigues *et al.* (2003) who reported adding 5.5 g L⁻¹ agar to the culture medium as compared to the normal concentration of 7-8 g L⁻¹ agar. However, low agar concentrations can cause unfavorable verification which result in failure of normal plant growth and may cause plantlet death. To avoid this phenomenon, 7 g L⁻¹ agar was used in the current study.

Shoot elongation: The analysis of variance of the data collected 8 week after culture initiation also indicated that the length of resultant shoots was significantly affected by an interaction between BA and IBA concentrations (Table 2). The effect of the tested hormonal combinations on the elongation of shoots derived from the node culture of Hassawi peach is shown in Fig. 3. The highest shoot length (4.74 cm) was associated with the medium containing 0.5 mg L⁻¹ BA and 0.1 mg L⁻¹ IBA. It is worth noting that this is the same treatment that resulted in the highest multiplication rate expressed in shoot number per explant.

Table 2: Analysis of variance of the effect of BA and IBA on shoot stage derived from node culture of Hassawi peach

Factor	df	Shoot No.		Shoot length		Leaf No.	
		MS	p-value*	MS	p-value	MS	p-value
BA	3	25.5861	0.0001	27.2416	0.0001	391.0972	0.0001
IBA	2	62.9083	0.0001	31.9226	0.0001	296.6583	0.0001
BA×IBA	6	29.1861	0.0001	04.8968	0.0020	241.6806	0.0001
Error	108	2.9843	--	1.3049	--	30.0491	--

*p-values>0.05 are significant

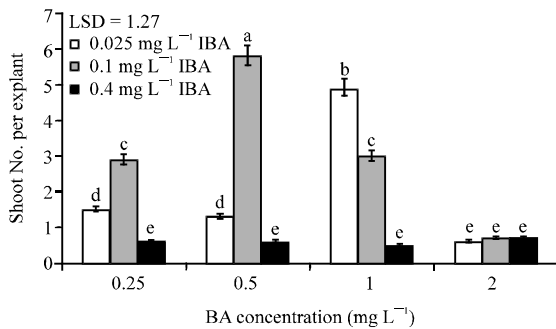


Fig. 2: Influence of BA and IBA concentrations on shoot number per axillary bud explant of Hassawi peach after 8 week of culture establishment

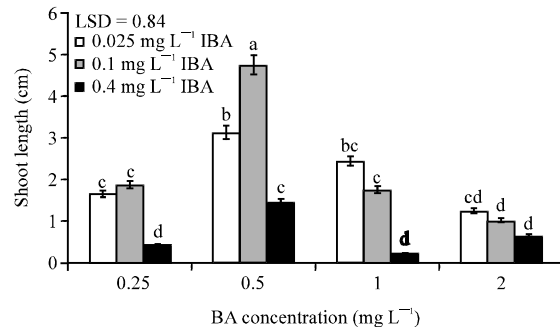


Fig. 3: Influence of BA and IBA concentrations on the length of shoots formed from axillary bud explant of Hassawi peach after 8 week of culture establishment

Similar results were reported by some researchers where combinations of IBA, BA were necessary for peach shoots development (Bhansali *et al.*, 1991; Zaied, 1997; Kassim *et al.*, 2010). Whereas, others noted that adding 5.8-11.4 μM gibberellic acid (GA_3) further promoted shoot elongation (Biddington, 1992). In contrast, GA_3 was found to have no pronounced effect on peach shoot elongation, proliferation, greening and rooting however, necrosis was decreased when 0.5-1 mg L^{-1} GA_3 was added to the culture medium (El-Deen *et al.*, 1998). In our study, sufficient elongation was achieved without the addition of GA_3 to the culture medium. However, future experimentation is necessary to determine the effect of GA_3 on this particular genotype.

Leaf formation: As the case with the other examined parameters, the analysis of variance revealed a significant interaction between BA and IBA concentrations affecting the number of leaves per shoot (Table 2). The effect of the hormonal treatments on the number of leaves per *in vitro* derived shoot of Hassawi peach is demonstrated in Fig. 4. The best result (19.3 leaves per shoot), was obtained by the same treatment that was optimum for both multiplication and elongation; namely, 0.5 mg L^{-1} BA and 0.1 mg L^{-1} IBA.

Rooting and *ex vitro* plant survival: Root formation was visible within 3 weeks after transferring the regenerated shoots to the 1/2 strength MS rooting media containing IBA. To ensure *ex vitro* survival the roots were allowed to grow for additional 3 week to allow sufficient root growth (Fig. 1d). The effect of four different concentrations of IBA on rooting percentage regenerated shoots of Hassawi peach after 6 week on rooting medium is illustrated in Table 3. In the absence of IBA root formation failed; whereas, media contained 0.25-1 mg L^{-1} IBA induced root formation but at different efficiency depending on the concentration. The highest rooting percentage (92.8%) occurred with 0.5 mg L^{-1} IBA. This treatment also gave the highest average number of roots (4.4 roots per shoot) and maximum root length (6.8 cm). Our finding is in harmony with previous reports (Perez-Tornero *et al.*, 2000; Ainsley *et al.*, 2001; Ahmad *et al.*, 2003).

However, (Ruzic *et al.*, 1984) mentioned that all the shootlets of peach x almond hybrid formed roots within 3 weeks in the presence of 1 mg L^{-1} NAA. Whereas (Marcelo *et al.*, 2003) and (Younas *et al.*, 2008) reported that the best root system was developed on 1/2 strength MS medium supplemented with 3 mg L^{-1} IBA but at a higher level induced callus formation and

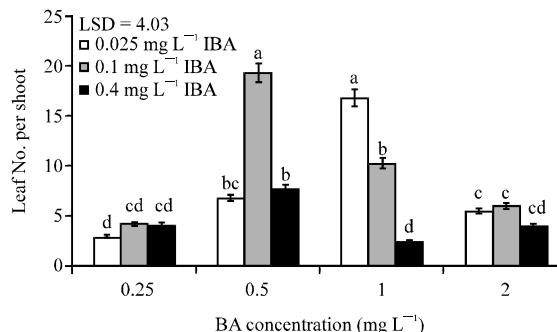


Fig. 4: Influence of BA and IBA concentrations on the number of leaves per shoot developed from axillary bud explant of Hassawi peach after 8 week of culture establishment

Table 3: Effect of IBA concentration supplied to the rooting media on Hassawi peach

IBA	Rooting (%)	Root No.	Root length (cm)
0	0 ^f	0 ^f	0 ^f
0.25	54.5±5.6 ^b	2.6±0.5 ^b	3.2±0.6 ^b
0.5	92.8±9.4 ^a	4.4±0.6 ^a	6.8±0.8 ^a
1	60.6±6.8 ^b	1.8±0.4 ^c	2.7±0.5 ^b

Numbers within the same column followed by different letters are significantly different

inhibited normal root development. Similarly, other researchers found that 1/2 strength MS medium gave the highest rooting rate and average number and lengths of root of peach (Ainsley *et al.*, 2001; Ahmad *et al.*, 2003; Fotopoulos and Sotiropoulos, 2005).

Acclimatization of peach using a mixture of equal amounts of peat moss, sand and vermiculite gave 95% transplant survival after 60 days. Plants growth was normal without any phenotypic deformities. This soil mixture was reported effective in the acclimatization of other plant species such as mulberry (El-Kazzaz *et al.*, 1997), almond (Zaied, 1997; Saeed, 1998) and black plum (Jain and Babbar, 2000).

In conclusion, this study points to the fact that selection of plant growth regulators play an important role in regulation of peach shoot regeneration. This is also true in other plant species (Siwach *et al.*, 2011; Silvarajan *et al.*, 2012). The concentrations of plant growth regulator as well as combinations were considered in this study which has determined a suitable treatment for Hassawi peach regeneration. This regeneration system can contribute to the conservation efforts towards preventing the extinction of this well adapted peach germplasm.

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