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IN VITRO PLANT REGENERATION OF TURKISH SWEET BASİL (*OCIMUM BASILICUM* L.)

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ANSTRACT

Sweet Basil (*Ocimum basilicum* L.) is most important medicinal and aromatic plant used in aromatherapy, cosmetics, perfume and food products in fresh or dried form. The present study provides a reliable and reaptable *in vitro* plant regeneration protocol of cultivated sweet basil of Turkey. Basil seeds were surface sterilized with 2.5% NaOCl. Epicotyl, hypocotyl and shoot tip explants were isolated from 12-14 days old *in vitro* grown seedlings. The explants were cultured on MS medium containing 0.80-2.40 mg/L TDZ with or without 0.10 mg/L IBA alongwith 1.0 mg/L PVP and 3.0 g/L activated charcoal. Cent percent callus induction were recorded on all explants on all culture mediums. Shoot regeneration frequency of epicotyl, hypocotyl and shoot tip explant ranged 75.0-100, 25.0-83.33 and 66.67-100% respectively. Maximum number of shoots from epicotyl (3.22) and shoot tip (3.58) were scored on MS medium containing 2.40 mg/l TDZ-0.10 mg/l IBA. Whereas, hypocotyl explant induced maximum number of (5.17) shoots per explant on MS medium with 2.0 mg/l TDZ. Relatively shorter roots of less than 1.0 cm were recorded irrespective of explant type or culture medium. However, shoots were successfully rooted and plantlets were transferred to pots and acclimatized well in growth room conditions.

Key words: *In vitro*, Epicotyl, Hypocotyl, Shoot tip, Shoot regeneration, Sweet basil.

INTRODUCTION

Sweet Basil (*Ocimum basilicum* L.) is a native plant of India (Viera and Simon 2000) and has been found in tropical and temperate regions of South Asia. The genus Ocimum shows distribution in Asia, Africa and Central America (Darrah 1998) and is cultivated in many European countries like France, Italy and Spain. In Turkey, basil is grown in Eastern Anatoian region as medicinal and aromatic plant as weel used in Turkish cuisine. Sweet basil shows wide range of variations due to its morphological (Simon *et al.* 1999; Labra *et al.* 2004 Nurzyńska-Wierdak 2007a), chemical contents and composition of essential oil (Marotti *et al.* 1996; Vieira and Simon 2000; Sifola and Barbieri 2006; Nurzyńska-Wierdak 2007b; Dzida 2010) as well as other biologically active substances (Golcz *et al.* 2006; Nguyen et al. 2010).

Basil is a valuable medicinal and aromatic plant mainly due to its essential oils obtained from leaves and flowers (Nurzynska-Wierdak *et al.* 2013) containing phenolic compounds (Simon *et al.* 1990; Phippen and Simon 2000) and other natural products including polyphenols such as flavonoids and anthocyanins (Phippen and Simon 1998). The compond 1.8 cineole, linalool, methyl chavicol (estragole) and eugenol (Charles and Simon, 1992; Marotti *et al.* 1996; Tohti *et al.* 2006) are the most important aroma components found in basil. Furthermore, monoterpenes (ocimene, geraniol, camphor), phenylpropanoids (methyl cinnamate,methyl eugenol) and sesquiterpenes (bisabolene, caryophyllene) are also present at difeerent concentrations in basil cultivars that influence the flavor (Charles and Simon 1992; Martins *et al.* 1999; Viera and Simon 2000). Phenol derivatives (eugenol, methyl eugenol, estragole, chavicol and methyl cinnamate) has also been reported in sweet basil (Werker *et al.* 1993; Miele *et al.* 2001).

Fresh basil is used as flavor foods such as poultry, vegetables and fish (Makri and Kintzois 2008). Use of basil as antioxidants (Bassiouny *et al.* 1990; Rice-Evans *et al.* 1997), insecticidal activity (Deshpande and Tipnis, 1997), antimutagenic activity (Stajković *et al.* 2007), antimicrobial (Koba *et al.* 2009) and antifungal (Dambolena *et al.* 2010) also make this plant important.

In vitro micropropagation of medicinal plants using different explants through tissue culture techniques provides an alternative way of isolation of secondary metabolites from *in vitro* induced calli or whole plant regeneration. It is also possible to enhance or alter the metabolite concentrations through *in vitro* cultures. Keeping in view, the present study was depicted to develop trustworthy and repeatable *in vitro* whole plant regeneration protocol alongwith acclimatisation of *in vitro* regenerated plantlets of medicianl sweet basil plant in order to use for secondary metabolites isolation.

MATERIALS AND METHODS

The seeds of Sweet basil were gained from Directorate General of Agriculture, Ankara, Turkey. The

seeds were surface sterilized with 2.5 % NaOCl (commercial bleach) for 10 min. Therefter, they were washed by sterile distilled water by continuos rinsing for 3x5 min. After sterilization, seeds were cultured on MS medium (Murashige and Skoog 1962) with 3.0 % sucrose and 0.65 % agar for germination. For plant regeneration, epicotyl, hypocotyl and shoot tip explant were isolated from 12-14 days old *in vitro* grown seedlings followed by inoculated on MS medium having 0.80-2.40 mg/L Thidiazuron (TDZ) singly or with 0.10 mg/L indole-3-butyric acid (IBA). Culture medium was also provided with 1.0 mg/L Polyvinylpyrrolidine (PVP) and 3.0 g/L activated charcoal in order to control necrosis due to phenolic compounds.

All culture media were autoclaved for 20 min at 1.4 kg cm^{-2}. The pH of all culture medium was adjusted to 5.6-5.8 using 1N NaOH or 1N HCl after adding TDZ and activated charcoal. All cultures (germination, plant regeneration and rooting) were grown at 24± 2°C with a 16/8-h (light/dark) photoperiod using cool-white fluorescent lamps with light intensity of 25 µmol $m^{-2}s^{-1}$. For rooting, *in vitro* regenerated shoots were isolated catrefully under aseptic conditions and cultured on MS medium with 0.20-1 mg/L IBA and data were scored after four weeks. For acclimatisation, the rooted plantlets were cleaned under tap water, followed by submerged into tap water for 15 min prior to transfer to pots containing 100% oraganic matter. All pots were covered with polyethylene bags for 10-12 days and kept under greenhouse condition.

The experiment was run in triplicate containing 8 explants each (6 x 8 = 48 explants). The data was analyzed using one way ANOVA with SPSS for Windows v. 17, SPSS, USA. Duncan's Multiple Range test was used for performing post hoc tests. The data transformed into percentages and arranged as arcsine transformation (Snedecor and Cochran 1967) prior to statistical analysis.

RESULTS

In the present study, epicotyl, hypocotyl and shoot tip explants were excised and inoculated on MS medium having 0.80-2.40 mg/L TDZ alone or with 0.10 mg/L IBA. Culture medium was also enriched with 1.0 mg/L PVP and activated charcoal and exerted positive effects on explants which did not show any sign of necrosis. It was intrestingly noted that due to higher concentrations of TDZ, all cultured explants induced high frequency of callus induction irrespective of IBA or explant type within two weeks followed by shoot induction with clear shoots after four weeks of culture (Fig 1 a, b, c). The data were noted on shoot regeneration, number of shoots per explant and mean shoot length and subjected to statistical analysis after 8 weeks of culture (Fig 2a). The analysis of variance of the results showed significant effects of TDZ-IBA on frequency of shoot regeneration (Table 1), number of shoots per explant (Table 2) and mean shoot length (Table 3). However, frequency of callus induction was found insignificant on all culture mediums (data not shown).

The frequency (%) of shoot regeneration showed the possitive effects of TDZ-IBA concentrations and found stasticscally significant for hypocotyl and shoot tip explants. Whereas, epicotyl explant showed insignificant level to TDZ-IBA concentrations (Table 1) and ranged 75-100 %. Shoot regenration frequency of hypocotyl and shoot tip explant ranged 25-83.33 % (Table 1) and 66.67-100% respectively (Table 1). Results further revealed that presence of IBA in the culture media controlled the shoot regeneration potential of individual explant. Shoot regeneration frequency of hypocotyl explant decreased with increase of TDZ concentration without supplemented IBA. Whereas, inclusion of 0.10 mg/l IBA increase the shoot regeneration frequency on same explant. On the other hand, shoot tip explant responded variably on culture media without IBA and ranged 66.67-100%. Whereas, epicotyl explant responded insignificant on TDZ-IBA containing media, it was recorded 83.33-100. It was intrestangly noted that shoot regeneration frequency influnced by TDZ-IBA combination in cultured medium compared to medium containing TDZ singly. Comparing response of explant type to culture media, shoot regeneration frequency of hypocotyl explant was generally low comapred to other explants.

As far shoots number per explants concerned, TDZ-IBA concentration was more effective on epicotyl and shoot tip explants, whereas, hypocotyl explant induced lower number of shoot per explant compare to TDZ singly in the present study. The maximum number of shoots per explant were 3.22, 5.17 and 3.58 noted on epicotyl, hypocotyl and shoot tip explant that ranged 1.83-3.22, 1.0-5.17 and 1.55-3.58 respectively (Table 2). Furthermore, the results indicated positive effects of IBA inclusion in culture medium with TDZ on number of shoots per explant of epicotyl and shoot tip explant and relatively more number of shoots per explant were observed. It was noted that the concentration of 1.60 mg/l TDZ inhibited shoots number per plant on epicotyl and shoot tip. Whereas, it was intermediate respone on hypocotyl explant on same concentration. On the other hand, TDZ alone produced more number of shoots per explant of hypocotyl explant compared to MS medium having TDZ-IBA and promotary effect of shoots per explant were observed on MS medium with 2 mg/l TDZ alone (Table 2). However, MS medium having 2 mg/l TDZ were also promotory for epicotyl and shoot tip explant.

Results on mean shoot length of all explants were found significant and showed inhibitory effects of higher concentration of TDZ in the culture medium as relatively shorter shoots below 1.0 cm were obtained irrespective of

IBA or explant type. Shoot length of epicotyl, hypocotyl and shoot tip explants ranged 0.27-0.85 cm, 0.22-0.79 cm and 0.39-0.94 cm respectively. Maximum shoot length from epicotyl (0.85 cm) and shoot tip explant (0.94 cm) were scored on MS medium containing 2.40 mg/L TDZ+0.10 mg/L IBA (Table 3). Whereas, maximum of 0.94 cm shoot length of hypocotyl explant was recorded on MS medium containing 0.80 mg/L TDZ+0.10 mg/L IBA. Results further illustrated that 0.10 mg/l IBA exerted positive effects on shoot length with TDZ concentration of 1.60 mg/l or above. Whereas, shorter shoots were obtained with lower concentration of TDZ used in the study irrespective of explant type.Comparing explant type, relatively longer shoots were obtained from shoot tip explant compared to other explants used in the study.

Irrespective of shorter *in vitro* regenerated shoots, they were isolated under aseptic conditions, inoculated on rooting medium having 0.25-1.00 mg/L IBA solidified with agar. Root initiation started within one week and 100% rooting (Fig 2b) was recorded on all rooting mediums after 4 weeks of culture. After 4 weeks, rooted plantlets were taken carefully and washed under tap water for removing agar in the rooting zone. Thereafter, rooted plants were placed in water for 15-20 min prior to transfer to pots containing substrates and kept in growthroom at room temperature for their growth. Approximately, 80.0% plants survived (Fig 2c) and continued their growth and set seed in growthroom conditions.

Table 1. Response of different explants of sweet basil (*Ocimum bascillicum* L.) on frequency of shoot regeneration on different concentrations of TDZ-IBA

TDZ (mg/l)	IBA (mg/l)	Frequency of Shoot Regeneration (%)		
		Epicotyl	Hypocotyl	Shoot tip
0.80	_	91.67^{ns}	58.33^{abc}	75.00^{bc}
1.20	_	100.00	58.33^{abc}	100.00
1.60	_	75.00	41.67^{bc}	66.67^{c}
2.00	_	91.67	41.67^{bc}	100.00
2.40	_	83.33	41.67^{bc}	91.67^{ab}
0.80	**0.10**	100.00	25.00^{c}	83.33^{abc}
1.20	**0.10**	83.33	50.00^{abc}	100.00^{a}
1.60	**0.10**	100.00	66.67^{ab}	100.00^{a}
2.00	**0.10**	100.00	83.33^{a}	100.00^{a}
2.40	**0.10**	83.33	75.00^{ab}	100.00^{a}

Means followed by different small letters within columns are sigificantly different using Duncan test at P<0.05

Table 2. Response of different explants of sweet basil (*Ocimum bascillicum* L.) on number of shoots per expant on different concentrations of TDZ-IBA

TDZ (mg/l)	IBA (mg/l)	Shoots per explant		
		Epicotyl	Hypocotyl	Shoot tip
0.80	_	2.89^{ab}	3.39^{ab}	2.03^{ab}
1.20	_	2.25^{ab}	2.55^{b}	2.17^{ab}
1.60	_	1.83^{b}	1.33^{b}	1.55^{c}
2.00	_	2.33^{ab}	5.17^{a}	3.17^{ab}
2.40	_	2.33^{ab}	1.67^{b}	2.28^{ab}
0.80	**0.10**	1.83^{b}	1.00^{b}	2.39^{ab}
1.20	**0.10**	2.92^{ab}	1.00^{b}	2.67^{ab}
1.60	**0.10**	2.17^{ab}	1.42^{b}	2.67^{ab}
2.00	**0.10**	3.08^{ab}	1.53^{b}	2.77^{ab}
2.40	**0.10**	3.22^{ab}	1.95^{b}	3.58^{a}

Means followed by different small letters within columns are sigificantly different using Duncan test at P<0.05

Table 3. Response of different explants of sweet basil (*Ocimum bascillicum* L.) on mean shoot length on different concentrations of TDZ-IBA

TDZ (mg/l)	IBA (mg/l)	Shoot Length (cm)		
		Epicotyl	Hypocotyl	Shoot tip
0.80	**_**	0.85[a]	0.65 [a]	0.94 [a]
1.20	**_**	0.56 [abc]	0.79 [a]	0.93 [a]
1.60	**_**	0.48 [abc]	0.18 [b]	0.41[b]
2.00	**_**	0.33[c]	0.22 [b]	0.65 [ab]
2.40	**_**	0.27[c]	0.22 [b]	0.39 [b]
0.80	**0.10**	0.42 [abc]	0.63 [a]	0.40 [b]
1.20	**0.10**	0.80[ab]	0.50 [ab]	0.60 [ab]
1.60	**0.10**	0.55 [abc]	0.50 [ab]	0.50 [ab]
2.00	**0.10**	0.51 [abc]	0.49 [ab]	0.43 [ab]
2.40	**0.10**	0.59 [abc]	0.58 [ab]	0.58 [ab]

Means followed by different small letters within columns are sigificantly different using Duncan test at P<0.05

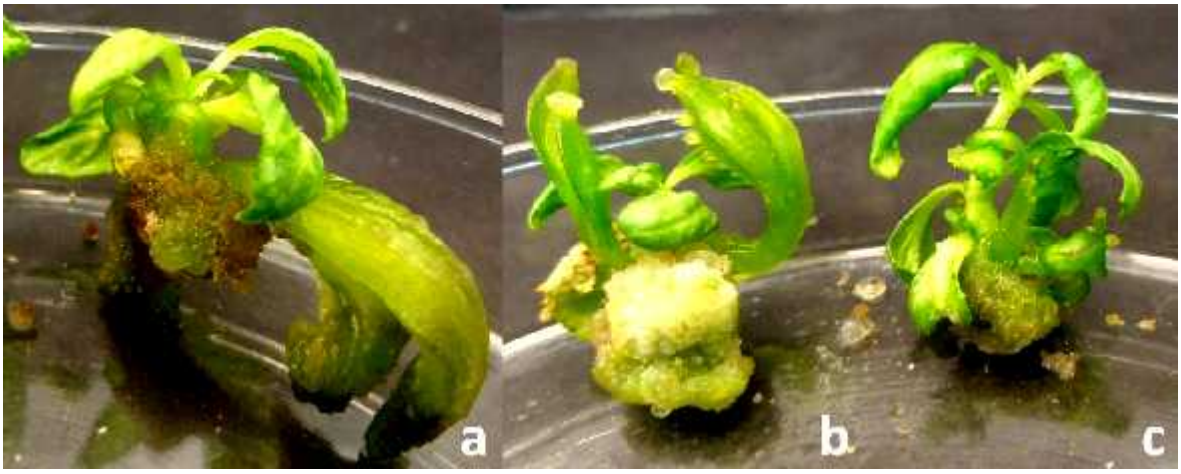

Figure 1 *In vitro* shoot regeneration from (a) epicotyl, (b) hypocotyl and (c) shoot tip explant of Antalya broad leaf basil on different concentrations of TDZ-IBA after 4 weeks of culture

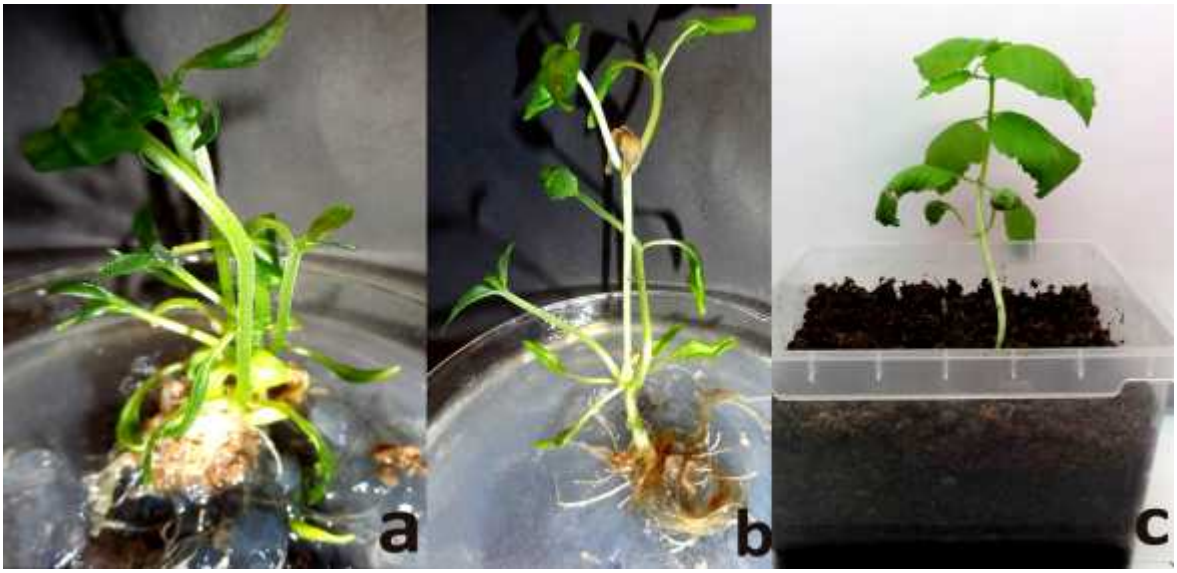

Figure 2 *In vitro* shoot regeneration, rooting and acclimatisation of Aantalya broad leaf basil (a) regenerated shoot from shoot tip after 8 weeks of culture, (b) rooted plantlet and (c) acclimatised basil plant in pot

DISCUSSION

In vitro micropropagation of medicinal plants (Shohael *et al.* 2006; Shin *et al.* 2008; Jeong *et al.* 2009; Park *et al.* 2012) with the objective to isolate secondary metabolites or altering or enhancing the concentration of secondary metabolites (Schijlen *et al.* 2006, Dorais *et al.* 2008) has been established. Although, *in vitro* regeneration of sweet basil cultivars of different origin has been successfully regenerated (Sahoo *et al.* 1997; Phippen and Simon, 2000; Siddique and Anis 2008; Saha *et al.* 2010), there is no report on the *in vitro* regeneration of basil cultivars cultivated in Turkey. This study presents the 1st ever report on successful shoot regeneration of basil cv. Antalya Broad Leaf using higher concentrations of TDZ. TDZ is one of the important cytokinin-like growth regulator (Thomas and Katterman 1986) used for *in vitro* shoot regeneration at relatively low concnetration (Sahin-Demirbag *et al.* 2008). However, our previous works on other basil cultivars using TDZ suggested the higher concentration requirement of TDZ for multiple shoot induction (Data not Shown). Therefore, in this study, relatively higher concentrations of TDZ (0.80-2.40 mg/l) were used singly or with 0.10 mg/l IBA for *in vitro* shoot regeneration of three different explants of sweet basil cv Antalya broad leaf. Similarly, Phibben and Simon (2000) also reported higher concentration of TDZ (16.8 µM or 4 mg/l) for maximum callus and shoot induction of basil.

Results revealed the different response of explants to TDZ-IBA concnetration on shoot regeneration frequency, shoots per explant and mean shoot length. However, all explants induced high frequency (100.0 %) of callus induction irrespective of TDZ concentration or availibility of IBA in the culture medium. Callus induction of many recalcitrant species including medicinal plants using TDZ and different explants has been reported (Mithila *et al.* 2003). Aasim *et al.* (2010) reported 100.0 % callus induction from cotyledonary node explant cultured on different concentration of TDZ-IBA in fenugreek. Similarly, Karatas *et al.* (2013) reported 100.0 % cllaus induction using TDZ-IBA in dwarf hygro. Contrarily, Basalma *et al.* (2008) reported suppressed callus induced by TDZ using hypocotyl and cotyledon explants of *Astragalus cicer*.

Results on shoot regeneration frequency showed the clear effects of explant type to TDZ-IBA concentration as shoot regeneration of epicotyl explant was found insignificant to growth regulators. In this study, hypocotyl and shoot tip explants showed sensitivity to growth regulators and higher concentration of TDZ induced more shoot regeneration frequency in line with Kaneda *et al.* (1997), who obtained maximum shoot regenration at 2.0 mg/l TDZ using cotyledonary node and hypocotyl explant of soybeans. In general, results revealed the positive effects of IBA with TDZ on shoot regeneration frequency in line with Çöçü *et al.* (2004) and Basalma *et al.* (2008). However, Aasim *et al.* (2010) reported suppressive effects of TDZ-IBA on shoot regeneration frequency of fenugreek. Comparing explant type, hypocotyl was less responsive than epicotyl or shoot tip possibly due to relatively more age or matured cells in line with Aasim *et al.* (2010). similarly, McLean and Nowak (1998) reported high shoot regeneration from hypocotyl compared to epicotyl explants of *Trifolium pratense.*

Results on number of shoots per explant also showed clear interaction between explant type, growth regulators and availibility of IBA in the culture medium. TDZ + auxin combinations regardless of concentrations and explant type has already been described for maximum number of shoots per explant of various plants (Anthony *et al.* 2004; Thambiraj and Paulsamy 2012). Results showed that all explants need higher concentration of TDZ for multiple shoot induction in line with Siddique and Anis (2008), who reported higher concentration of cytokinin requirement for shoot bud initiation at initial stage followed by lower cncentration in sweet basil. Our results further revealed that presence or absence of IBA affected the number of shoots per explant as 2.0 mg/l TDZ without IBA was found sufficient for maximum shoots from all explants. Contrarily, all explants gave maximum shoots on MS medium supplemented with 2.40 mg/L TDZ+0.10 mg/L IBA which showed that explants needs specific concentration of TDZ-IBA for maximum shoots in line with Siddique and Anis (2008) who also obtained maximum shoots per explant BA-IAA and KIN-IAA containing medium.

Higher concnetrations of TDZ inhibited the shoot length of regenerated plants from all explants. Reduced shoot length using TDZ at higher concentrations is well known phenomenon and has been reported previously (Huetteman and Preece 1993; Pattnaik and Chand 1996, Murch *et al.* 2000, Faisal *et al.* 2005; Ruzic and Vujovic 2008). The suppression caused by TDZ on shoot length that might be consistent with its high cytokinin activity (Huetteman and Preece 1993). Results clearly illustrated the effects of higher concentration of TDZ and relatively lower TDZ concentrations is found sufficient for maximum shoot length. Similarly, Sahin-Demirbag *et al.* (2008) gained longer shoots at 0.05 mg/l TDZ in Hungarian vetch. Kendir *et al.* (2009) reported shorter plants of Narbon vetch at higher concentration of TDZ. Lata et al. (2009) also reported similar observations in *Cannabis sativa* (L). Results further illustrated that IBA with TDZ has positive efefcts on mean shoot length with higher concentrations of TDZ in line with Aasim *et al.* (2010, 2011) and Kendir *et al.* (2009). However, with low concentrations used in the present study, IBA with TDZ hindered the shoot length. Presence of IBA influenced positively on shoot length with all growth

regulators in line with Brar *et al.* (1997) and Karataş *et al.* (2013).

Higher concentration of TDZ is oftenly associated with low rooting due to suppressory effects of TDZ in the culture medium and has been described for lentil (Fratini and Ruiz 2002; Khawar *et al.* 2004), *Cercis canadensis* L.var. alba (Rehder) Bean. (Yusnita *et al*., 1990), *Hibiscusrosa sinensis* L. (Preece *et al.* 1987) and in muscadine grape (Gray and Benton 1991). However, in this study, *in vitro* regenerated shoots were successfully rooted at various concentrations of IBA irrespective of higher concentrations of TDZ used for the regeneration. Rooted plantlets dipped in water for 15-20 prior to transfer to pots to avoid wilting is in line with Aasim (2012) and Aasim et al. (2013) and resulted in successfully acclimatised in pots under growth room conditions and survival rate of more than 80.0%.

The successful development of *in vitro* regenration and acclimatisation of important medicinal plant establish a new vista in basil biotechnology and can be employ for isolation of secondary metabolites or genetic transformation studies using established protocol.

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