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DIRECT AXILLARY SHOOT REGENERATION FROM THE MATURE SEED EXPLANT OF THE HAIRY VETCH (*VICIA VILLOSA* ROTH)

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Abstract – The hairy vetch (*Vicia villosa* Roth) is a climbing, prostrate or trailing legume grown as forage.It fixes atmospheric nitrogen, reduces soil erosion and provides an instant mulch. Multiple axillary shoot regeneration from a mature seed explant (zygotic embryo with two cotyledons) was obtained on MS medium containing 0.05 – 1.6 mg/l TDZ with or without 0.10 mg/l IBA. The frequency (%) of shoot regeneration ranged from 45.83-75.00% with a maximum number of 28.6 shoots per explant on MS medium containing 0.20 mg/l TDZ-0.10 mg/l IBA. The mean shoot length decreased proportionately with each increase in TDZ concentration irrespective of the IBA concentration in the culture medium. However, comparing the two types of regeneration media, longer shoots were recorded in the presence of IBA in the culture medium. Regenerated shoots were pulse treated with 50 mg/l IBA for 5, 10 and 20 min for rooting.

Key words: *In vitro*, legume, pulse treatment, rooting

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INTRODUCTION

The hairy vetch (*Vicia villosa*), also known as fodder vetch or winter vetch, is an important fodder legume grown as a forage crop and as a companion plant with tomato (Abdul-Baki and Teasdale, 1993). It is native to Europe and western Asia. It is a climbing, prostrate or trailing legume and has good tolerance to adverse soils; it can be grown under low or high rainfall conditions in a wide pH range of 4.9 to 8.2 (Duke and James, 1990). It can fix atmospheric nitrogen in the range of 45-180 pounds per acre (Smith et al., 1987) and reduce soil erosion, providing an instant mulch that preserves moisture and keeps weeds from sprouting (Verhallen et al., 2003).

Successful *in vitro* shoot regeneration has been reported in other vetches, such as the narbon vetch (Donn, 1978; Roupakias, 1985; Pickardt and Schieder, 1987; Pickardt et al., 1989; Albrecht and Kohlenbach, 1989; Tegeder et al., 1996; Kendir et al., 2008, 2009), Hungarian vetch (Sancak et al., 2000; Sahin-Demirbag et al., 2008) and faba bean (Fakhrai et al., 1989; Khalafalla and Kazumi, 1999). However, to the best of our knowledge, to date there is no report available on *in vitro* shoot regeneration in the hairy vetch.

The objective of the study was to induce rapid and repeatable axillary shoot regeneration from the mature seed explant of the forage legume hairy vetch, for further use in breeding studies.

MATERIALS AND METHODS

The seeds of *V. villosa* were obtained from the Osman Tosun Gene Bank, the Department of Field Crops, Faculty of Agriculture, Ankara University,

Ankara, Turkey. The seeds were surface-sterilized with 100% commercial bleach (Ace-Turkey containing 5% NaOCl in a laminar flow hood by continuous stirring for 20 min. This was followed by 3 x 5 min rinsing with sterile distilled water. Thereafter, mature seeds with intact test (zygotic embryo with two cotyledons) were cultured on MS medium containing 3% sucrose, 3 g/l activated charcoal supplemented with 0.05 to 1.6 mg/l TDZ with 0.0 or 0.10 mg/l indole-3-butyric acid (IBA) in GA7 magenta vessels™. All cultures were incubated at 24±2°C in a 16 h day length photoperiod. The pH of all cultures was adjusted to 5.6 – 5.8 before adding 0.65% agar (Duchefa) and autoclaving at 121°C, 118 kPa pressure for 20 min.

After 8 weeks of culture, the data regarding the frequency (%) of shoot regeneration, the number of shoots per explant and shoot lengths were recorded. Thereafter, well regenerated shoots were pulse-treated with 50 mg/l IBA for 10 min for rooting. Rooted shoots were transferred to a potting mixture containing equal ratios of peat moss vermiculite and perlite. Plants in plastic pots were covered with polyethylene bags for 1 week and maintained in a greenhouse under ambient conditions of temperature and natural light. They were watered every 2 days for 2 weeks.

Each treatment had 6 replicates containing 12 explants each (3 x 8 x 2 = 48 explants). The data was analyzed with SPSS 17.0 using one way ANOVA; the post hoc tests were performed using Duncan's Multiple Range test. Data given in percentages were subjected to arcsine transformation (Snedecor and Cochran, 1967) before statistical analysis.

RESULTS

Explants showed swellings after 5-7 days of culture on MS medium containing TDZ irrespective of the presence or absence of IBA in the culture media, followed by single shoot regeneration in 10-12 days. Multiple shoot regeneration without callus induction started from the embryonic axis after three weeks of culture (Fig 2a). No shoot regeneration was observed from the cotyledons (Fig 1a). Callus induction started late and was recorded only on the radicle part of the explant (Fig 2a). Single shoot regeneration was recorded on an MS medium (control).

Analysis of variance results showed insignificant effects of plant growth regulators on shoot regeneration frequency (45.83-75.00%). Maximum shoot regeneration was recorded on the MS medium supplemented with 0.05 mg/l TDZ-0.10 mg/l IBA (Table 1). The results showed that the presence of IBA in the culture medium increased shoot regeneration compared to culture medium without IBA. Analysis of variance results further showed significant varia-

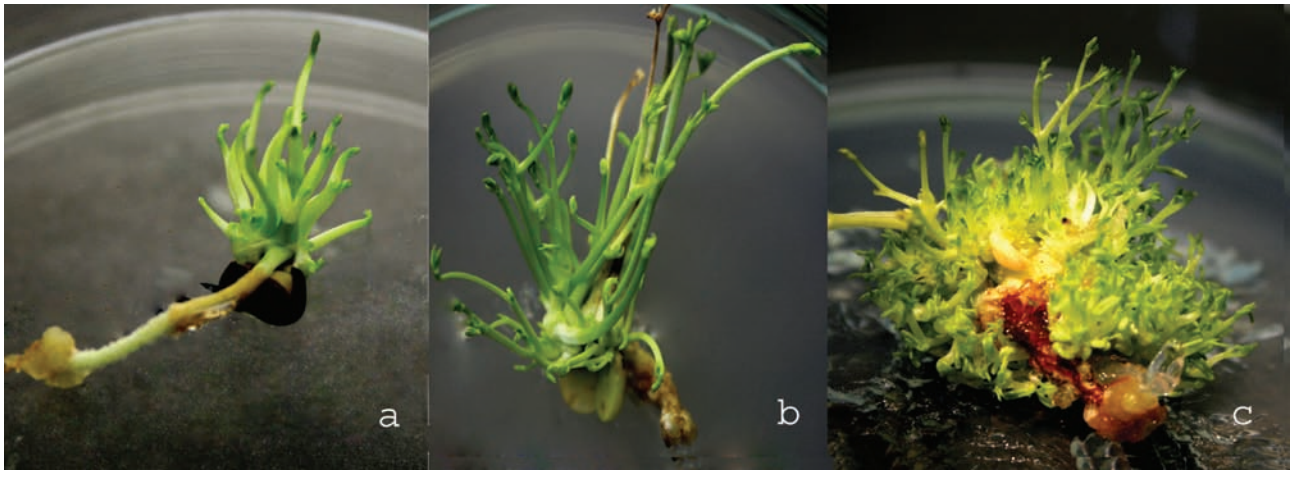

Fig. 1. Axillary shoot regeneration from a mature seed explant of hairy vetch (a) shoot initiation after 3 weeks; (b) shoot proliferation with visible cotyledons; (c) shoot regeneration after 6 weeks

Table 1. The effects of TDZ-IBA concentrations on shoot regeneration from a mature seed explant of the hairy vetch

TDZ (mg/l)	IBA (mg/l)	Frequency of shoot regeneration (%)	Mean number of shoots per explant	Shoot length (cm)
0.05	-	62.50^{ns}	25.07^{ab}	2.93^{ab}
0.10	-	62.50	20.78^{bc}	2.61^{bc}
0.20	-	62.50	17.64^{cd}	2.30^{cd}
0.40	-	50.00	25.00^{ab}	1.56 ^{e}
0.80	-	45.83	17.78^{cd}	1.57^{e}
1.60	-	58.33	15.72^{d}	1.90^{de}
0.05	0.10	75.00	14.40^{d}	3.31^{a}
0.10	0.10	62.50	23.47^{b}	2.42 ^{bcd}
0.20	0.10	62.50	28.60^{a}	2.60^{bc}
0.40	0.10	54.17	21.14^{bc}	2.42^{bcd}
0.80	0.10	50.00	17.75^{cd}	1.87^{de}
1.60	0.10	54.17	15.40^{d}	2.71^{bc}

Values in a column followed by different letters are significantly different according to Duncans Multiple range test

tion in the mean number of shoots per explant and mean shoot length. The mean number of shoots per explant showed inconsistent behavior and ranged from 14.40-28.60. The maximum number of 28.60 shoots per explants was recorded on the MS medium containing 0.20 mg/l TDZ-0.10 mg/l IBA followed closely by the MS medium containing 0.05 mg/l TDZ. The minimum number of 14.40 shoots was recorded on the MS medium containing 0.05 mg/l TDZ-0.10 mg/l IBA.

The mean shoot length decreased with the increase in TDZ concentration irrespective of IBA in the culture medium. However, IBA in the culture medium increased the average mean shoot length compared to MS medium devoid of IBA. The longest shoots of 3.31 cm were recorded on the MS medium containing 0.05 mg/l TDZ-0.10 mg/l IBA, followed by 2.93 cm-long shoots on the same TDZ concentration without IBA.

Regenerated shoots were pulse-treated with 50 mg/l IBA for 5, 10 and 20 min and transferred to an MS medium devoid of auxins in Magenta vessels or directly to pots containing organic matter. The pots were covered with plastic bags for 1 week. After 1 week, the plastic bags were removed and the pots were left in the greenhouse for acclimatization. No rooting was observed in either magenta vessels or pots.

DISCUSSION

Direct *in vitro* regeneration of hairy vetch plantlets under *in vitro* conditions from a mature embryo with two cotyledons (mature seed) explant is an important achievement for this important plant species. The protocol provides an alternative means for the improvement of hairy vetch through tissue culture. Direct shoot regeneration from a mature seed explant has been reported in other legumes such as the chickpea (Polisetty et al*.,* 1997), mung bean (Harisaranraj et al*.,* 2008), peanut (Li et al., 1994; Cucco and Juame, 2000; Gagliardi et al., 2000; Palanivel and Jayabalan 2002; Pacheco et al., 2007), narbon vetch (Kendir et al., 2009); in cereals like rice (Masaaki et al., 2004; Bano et al., 2005) and wheat (Malik et al., 2004), and in other plant species like *Epimedium alpinum* L, (Mihaljević and Vršek, 2008), onion (Khar et al., 2005) and spinach (AL-Khayri et al., 1992). To the best of our knowledge there is no report on the *in vitro* shoot regeneration of hairy vetch. This report provides a simple and efficient shoot regeneration of hairy vetch using a mature embryo with two cotyledons (mature seed) explant.

Single shoot regeneration from control experiments showed the clear bearings of plant growth regulators on multiple shoot regeneration from mature seed explants of the hairy vetch under *in vitro* conditions. The results showed that an MS medium containing different concentrations of TDZ-IBA induced callus on the radicle part of the explant in line with Harisaranraj et al. (2008) who reported callus induction from half seed explants on media containing cytokinins and auxins in mung bean. Similar results of callus induction were also reported in wheat (Malik et al., 2004), onion (Khar et al., 2005) and rice (Bano et al., 2005). Contrarily, Kendir et al. (2009) reported no effect of plant growth regulators on callus induction in narbon vetch using mature seed explants.

Single shoot regeneration started from the embryonic axis of the seed explants within 10-12 days of culture, followed by multiple axillary shoot regeneration after 3 weeks of culture. Polisetty et al. (1997) also achieved shoot/shoot bud differentiation in 45–90 days from a mature embryo with two cotyledons (mature seed) explant of chickpea. Pacheco et al. (2007) reported single shoot development from the embryonic axes of the peanut in response to 4.4 μM BAP. However, Gagliardi et al. (2000) reported the failure of a mature embryo with two cotyledons (mature seed) to germinate on MS either without growth regulators (MS0) or supplemented with 10 μM TDZ in peanut. Multiple shoot regeneration from embryonic axes with no regeneration from cotyledons is in line with Kendir et al. (2009).

The results further showed that variable concentrations of TDZ-IBA in the culture medium had no effect on shoot regeneration frequency, which is in agreement with Kendir et al. (2009), who reported no effect of BAP concentration on the shoot regeneration frequency of a mature seed of Narbon vetch. The results further showed that the presence of IBA in the culture media did not affect shoot regeneration behavior but generally increased the average shoot regeneration. Sahin-Demirbag et al. (2008) reported an increase in shoot regeneration frequency with each increase in TDZ concentration in Hungarian vetch, whereas, Aasim et al. (2009, 2010) reported shoot regeneration frequency decreased with the increase in TDZ concentration with 0.10 mg/l IBA in fenugreek

The results reflected the variable effects of TDZ-IBA concentrations on the mean number of shoots per explant. MS medium containing ≥ 0.40 mg/l TDZ, irrespective of the presence or absence of IBA in the culture medium, had a promotory effect on the number of shoots per explant, and a further increase of TDZ in the culture medium had an inhibitory effect on the mean number of shoots per explant. Sahin-Demirbag et al. (2008) also reported maximum shoot regeneration on an MS medium containing 0.45 mg/l TDZ and a further increase of TDZ concentration significantly inhibited shoot regeneration in the Hungarian vetch. The minimum number of shoots on the MS medium containing 0.05 mg/l TDZ-0.10 mg/l IBA might be due to an inhibitory effect of IBA in fenugreek in line with Aasim et al. (2009, 2010).

The results also showed the inhibitory effect of TDZ on mean shoot length, which decreased with each increase in TDZ concentration. Sahin-Demirbag et al. (2008) also reported a maximum shoot length at the lower concentration of 0.05 mg/l TDZ which reduced proportionately with each increase in the concentration of TDZ in Hungarian vetch. Kendir et al. (2009) also reported a decreased shoot length of the seed explant (zygotic embryo with two cotyledons) of the Narbon vetch with increasing BAP concentrations. The presence of IBA in the culture medium positively increased the mean shoot length compared to medium devoid of IBA in agreement with Aasim et al. (2009, 2010); who reported the positive effects of IBA or auxin in the culture media on the shoot length of fenugreek.

The regenerated shoots which were pulse-treated with IBA for 5, 10 and 20 min failed to induce rooting in Magenta vessels and in pots. The use of pulse treatment of IBA for rooting reports successful rooting in the narbon vetch (Kendir et al., 2008, 2009), the Hungarian vetch (Sahin-Demirbag et al., 2008;

Sancak et al., 2000) and Sainfoin (Sağlam, 2010). Further experiments to induce adventitious rooting of this important forage legume are underway.

The protocol will be useful for direct organogenesis by using mature seeds (embryos with two cotyledons) as explants. The protocol can be used in future for clonal multiplication and increasing the breeding activities of individual genotypes belonging to the *Vicia* species. Moreover, it is expected that the protocol could provide a potential for the genetic transformation of this important forage legume plant for improved plant characteristics.

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