Journal of Applied Biological Sciences 6 (2): 43-45, 2012
ISSN: 1307-1130, E-ISSN: 2146-0108, www.nobel.gen.tr

In Vitro Shoot Regeneration of Common Vetch (*Vicia sativa* L.) Using Immature Cotyledons

Muhammad AASIM

Biology Department, Kamil Ozdag Faculty Of Science, Karamanoglu Mehmetbey University, Karaman, Turkey

*Corresponding Author
e-mail: mshazim@gmail.com

Received: March 10, 2012
Accepted: April 12, 2012

Abstract

Common vetch (*Vicia sativa* L.) is multi usage legume as a cover crop, green manure, pasture, silage, and hay due to its high dry matter and nitrogen accumulation. It is neglected crop due to toxicity to non-ruminant animals including humans. It causes a disease called favisim due to the presence of an oxidants like convicine, isouramil, divicine and vicine which results in lowering of glutathione levels in G6PD-deficient persons. Immature cotyledon seeds were cultured on MS medium containing 0.05-0.80 mg/l TDZ-0.10 mg/l IBA. Callusing without shoot regeneration was observed only on higher concentarion of 0.80 mg/l TDZ-0.10 mg/l IBA in the culture medium. Shoot regeneration frequency and shoots per explants ranged 25.0-58.33% and 8.33-19.33 respectively. Maximum Shoot regeneration frequency (58.33%) and shoots per explants (19.33) were recorded on MS medium supplemented with 0.20 mg/l TDZ-0.10 mg/l IBA. Equal concentration of TDZ-IBA induced maximum shoot length and was recorded 5.0 cm. Most of the palnts rooted directly in the culture medium and remaining were cultured on MS medium containing 1.0 mg/l IBA. Both types of plantlets were acclimatized under ambient conditions.

Key Words: Common vetch, Immature cotyledons, In vitro, Shoot regeneration

INTRODUCTION

Common vetch (*Vicia sativa*), or simply "the vetch", is a succulent and annual nitrogen fixing legume used as a cover crop, green manure, pasture, silage, and hay. Its high dry matter and nitrogen accumulation, and the absence of hard seeds, make it an excellent winter leguminous cover crop in annual vegetable rotations and provide substantial amounts of N to the following crop when planted alone. It also offers excellent spring weed suppression and grows well in mixtures with cereal grains that can provide both cool-weather weed suppression and fall N scavenging [1] Common Vetch is a native of Europe, but also common in some parts of Northern Africa and Southwestern Asia.

Full potential of *Vicia* species has not been well established due to toxicity to non-ruminant animals including humans. Common Vetch (*V. sativa*) contains γ-glutamyl with active molecule of β-cyanoalanine; which inhibits the conversion of the sulfur amino acid methionine to cysteine leading to the production of Cystathionine, which is an intermediary product of this biochemical reaction and is secreted in urine [2]. This depletes vital protective reserves of the sulfur amino acid cysteine and make its seeds dangerous. Common Vetch along with broad bean are common cause of a disease called favisim due to the presence of an oxidants like convicine, isouramil, divicine and vicine which results in lowering of glutathione levels in G6PD-deficient persons.

The genus *Vicia* L. (Leguminosae, Vicieae) comprises about 166 species, but in vitro shoot regeneration been reported only in faba bean [3,4,5,6,7,8,9], narbon vetch [10,11,12,13], Hungarian vetch [14,15] and in Hajastana vetch [16]. However, no report covers the in vitro shoot regeneration of common vetch to date. This study presents the shoot regeneration protocol of common vetch in order to improve this neglected crop to be using modern biotechnological techniques in future.

MATERIAL AND METHODS

Green pods of common vetch were collected from experimental field of Department of Field Crops, Faculty of Agriculture, Ankara University, Ankara, Turkey. Pods were surface sterilized with 100% commercial bleach (Ace-Turkey containing 5% NaOCl) for 10 min. followed by 3x5 min. rinsing with bidistilled sterilized water for 5 min. Thereafter, the immature seeds were isolated under aseptic conditions and de-embryonated immature cotyledons were cultured on agar solifdified MS [17] basal medium containing 0.05, 0.10, 0.20, 0.40 and 0.80 mg/l TDZ (Thidiazuran) with 0.10 mg/l IBA (Table 1). Explants were also cultured on MS medium free of growth variants to serve as control. Medium was also supplemented with 1 mg/l Polyvinylpyrrolidine (PVP-average mol. wt. 10,000, cat No. P2307, Sigma Aldrich, St. Lo. MO.), 3.0% sucrose and 5 g/l activated charcoal. After 8 weeks of culture, explants were subcultured on same medium devoid of activated charcoal. Data for frequency (%) of callusing, frequency (%) of shoot regeneration, mean number of shoots per explant, mean shoot length was taken after 16 week of culture.

For rooting, regenerated shoots were cultured on rooting medium containing 1.0 mg/l indole 3 butyric acid (IBA- cat no. I5386, Sigma Aldrich Chemical Co. St. Lo. Mo) in Magenta

Table 1. Effects of MS medium containing variants of TDZ-IBA on shoot regeneration from immature cotyledons of common vetch

TDZ	IBA	Frequency (%) of Callusing	Frequency (%) of regeneration	Mean umber of shoots per explant	Shoot length (cm)
0.05	0.10	0.00b	25.00b	8.33c	3.67b
0.10	0.10	0.00b	41.67ab	13.33b	5.00a
0.20	0.10	0.00b	58.33a	19.33a	4.00ab
0.40	0.10	0.00b	41.67ab	11.67bc	4.67ab
0.80	0.10	33.33a	0.00c	0.00c	0.00c
0.00	0.00	0.00b	0.00c	0.00c	0.00c

[1]Values within column in a block followed by small letters are significantly different at the 0.05 level by Duncan's test.

GA7 vessels for six weeks. Thereafter, the rooted plantlets were removed carefully from the agar containing media under tap water and kept submerged in water for 10-15 min before transferring to pots containing organic matter+coarse grained sand (1.1). The pots were covered with transparent polythene bags for 2 weeks to avoid wilting and placed in growth room at ambient condition of temperature and humidity. After 10 days, holes were punched into the polythene bags to allow for acclimatization by decreasing relative humidity gradually. The polythene bags were completely removed after 15 days, once the plants showed the signs of growth and acclimatization.

The pH of all culture and rooting media was adjusted to 5.6 - 5.8 using 0.1 N KOH or 0.1 N HC1 before autoclaving at 118 kPa and 121□C for 20 minutes. Filter sterilized IBA were added to the culture media after autoclaving at 40-42□C. All cultures were incubated in growth room at 24 ± 2 °C with 16 h light photoperiod with low light intensity of ~ 5000 lux.

The experiment was designed as single factor experiment, including five treatments with three replications containing eight explants per replicate and was repeated twice (8 x 3 x 2 = 48 explants). Data for frequency (%) of callusing, frequency (%) of shoot regeneration, mean number of shoots per explant, mean shoot length was subjected to one way ANOVA using F test with statistical software SPSS 16.00 for windows. The post hoc tests were performed using Duncans Multiple Range Test (DMRT) to compare the differences between control and other treatments. Data given in percentages were subjected to arcsine transformation [18] before statistical analysis.

RESULTS

Swelling of explants started within 1 week of culture from embryonic end of immature cotyledon explant followed by nodal formation after 2 weeks. However, shoot regeneration was very slow and clear cut shoot regeneration was recorded after 4 weeks of culture on MS medium containing TDZ-IBA. No activity of callusing or nodal initiation was observed on explants cultured on MS medium free of growth regulators. Initial shoot regeneration followed by shoot initiation and elongation was very slow and subculturing after 8 weeks without activated charcoal exerted positive impact on shoot initiation and shoot elongation.

Callusing was observed only on explants cultured on MS medium containing 0.80 mg/l TDZ-0.10 mg/l IBA and recorded 33.33% (Table 1). Contrarily, no shoot regeneration was recorded on MS medium containing 0.80 mg/l TDZ-0.10 mg/l IBA. Frequency of shoot regeneration and mean number of shoots per explant ranged 0.0-58.33% and 0.0-19.33 respectively (Table 1). Maximum shoot regeneration frequency (58.33%) and mean number of shoots per explant (19.33) was recorded on MS medium containing 0.20 mg/l TDZ-0.10 mg/l IBA. Results on frequency (%) of shoot regeneration and mean number of shoots per explants showed similar trend and increased with increase in variants with maximum shoot regeneration and shoots per explants were recorded on MS medium containing 0.20 mg/l TDZ-0.10 mg/l IBA which was followed by decline and no shoot regeneration at higher concentration of 0.80 mg/l TDZ-0.10 mg/l IBA in the culture medium.

However, results on shoot length showed somewhat different trend and responded variably to TDZ-IBA combination. Shoot length ranged 3.67-5.00 cm (Table 1) with minimum shoot length of 3.67 cm and maximum shoot length of 5.00 cm was recorded on MS medium supplemented with 0.05 mg/l TDZ-0.10 mg/l IBA and 0.10 mg/l TDZ-0.10 mg/l IBA respectively.

Some of the regenerated shoots already rooted in the culture media and transferred directly to pots containing organic matter and sand where they acclimatized successfully. Whereas, shoots without roots were transferred to MS medium containing 1 mg/l IBA and 50% shoots were rooted after 6 week. Rooted plantlets were transferred to pots where most of the plants failed to survive but remained plants acclimatized well and set flowering under growth room conditions.

DISCUSSION

Results showed that explants responded well to growth variants at early stage but could not convert those shoot buds into proper shoots. However, sub culturing to the same growth variants devoid of activated charcoal which in turn not only increased the shoot proliferation but also enhanced the shoot elongation. This might be due to the presence of activated carbon in the culture medium. Activated charcoal acts both in promotion and inhibition of culture growth depending upon the plant species. Aasim et al. [19] reported positive effects of activated charcoal on shoot regeneration at early stage in cowpea.

Results showed clear relationship between callusing, shoot regeneration and growth variants concentration. Higher concentration of TDZ caused callusing on some explants

and totally inhibited the shoot regeneration. Whereas, other concentration of TDZ did not induce callusing. Aasim et al. [20,21] also reported low or no callusing in fenugreek cultured on TDZ-IBA containing medium. Similarly, Aasim et al. [22] also reported low callusing in hairy vetch cultured on TDZ-IBA containing medium.

Results further showed clear response of explants to the growth variants and MS medium supplemented with 0.20 mg/l TDZ- 0.10 mg/l IBA was found optimum medium for shoot regeneration and mean number of shoots per explant. Shoot regeneration and number of shoots per explant gradually increased with increase in variants concentration up to optimum concentration of 0.20 mg/l TDZ- 0.10 mg/l IBA. After that concentration, there was sharp decline of shoot regeneration frequency and mean shoot length followed by complete inhibition at 0.80 mg/l TDZ-IBA in the culture medium. Sahin-Demirbag et al. [15] obtained maximum number of shoots per explant on MS medium supplemented with 0.45 mg/l TDZ that was followed by sharp inhibition in Hungarian vetch.

Although, growth variants showed statistically significant on mean shoot length but equal concentration of TDZ and IBA was found more appropriate to get maximum shoot length compared to other concentrations. Sahin-Demirbag et al. [15] reported decreased shoot length with increase of TDZ concentration in the culture medium. This might be due to low light intensity and relatively longer culture time used for in vitro shoot regeneration.

Results also showed the development of whole plant regeneration in the culture medium. This phenomenon is very rare and these plants were not difficult to acclimatize under growth room conditions. Whereas, regenerated shoots were also rooted successfully at reasonable rate of 50% followed by successful acclimatization under ambient conditions. Şahin-Demirbag et al. [15] reported successful rooting of Hungarian vetch followed by acclimatisation. Contrarily, Aasim et al. [22] failed to root TDZ-IBA induced shoots of hairy vetch on MS medium supplemented with IBA concentrations.

Successful regeneration protocol is the prerequisite for common vetch which is the important forage legume of Turkey. This protocol provides reliable and repeatable protocol for common vetch multiplication and open a wide field for application of biotechnological tools like genetic transformation in the future.

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