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Araştırma Makalesi

Establishment of a Novel Plant Micropropagation System from Mature Hydroprimed Seeds of Two Turkish Broad Bean Cultivars

Ferzat TURAN[1*], Khalid Mahmood KHAWAR[2]

[1]Sakarya Applied Sciences University, Faculty of Agricultural Sciences, Field Crops Department, Sakarya, Türkiye
[2]Ankara University, Faculty of Agricultural Sciences, Field Crops Department, Ankara, Türkiye

[*]Corresponding Author: ferzatturan@subu.edu.tr

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Abstract

Leguminous Broad bean (*Vicia faba* L.) is an excellent source of minerals, vitamins and proteins that makes it highly valuable food for human and feed for animal consumption. The plant has an important characteristic to fix atmospheric Nitrogen and play an important role to establish a natural balance of N in the atmosphere. It is a highly self-pollinated plant and has problem of low variation with limited genetic pool. Moreover, the previous studies identify problem of recalcitrance in broad bean. Therefore, there is need to establish a repeatable micropropagation protocol that could ensure an increase in genetic variability to overwhelm problems in breeding. This system must also be able for efficient gene delivery and could be integrated with the conventional breeding programs through direct organogenesis. The study aimed to develop a tissue culture (shooting & rooting) protocol on two important Turkish broad bean cultivars Filiz99 and Eresen87 using MS medium containing 0.05, 0.15, 0.25, 0.35, 0.45, 0.55 mg l^{-1} TDZ using embryonic axis as explant. Maximum number of 5.33 and 3 shoots per explant were noted on cv. Filiz99 and Eresen87 on MS medium containing 0.15 mg l^{-1} TDZ. The developing shoots were rooted on MS medium containing 1 mg l^{-1} IAA after three weeks of culture. The rooted plants were transferred to pots containing peat under maintained under controlled greenhouse conditions for acclimatization The acclimatized plants bloomed and set seeds. Present results underscore importance of seed hydropriming before taking of explants to achieve high micropropagation on faba beans to overcome recalcitrance.

Key words*:* Faba bean, hydropriming, regeneration, rooting, Vicia faba

Türkiye'de yetiştirilen iki bakla Çeşidinin Olgun Tohumları Üzerine Hydropriming Uygulamasıyla Mikro-Çoğaltma Sisteminin Geliştirilmesi

Özet

Bakla (*Vicia faba* L.); mineral, vitamin ve protein kaynağı olmasından dolayı insan ve hayvan beslenmesi için son derece değerli bir bitkidir. Aynı zamanda atmosferdeki azotu sabitlemek gibi önemli bir özelliğe sahiptir ve bu yönüyle atmosferdeki doğal nitrojen dengesini kurmada önemli bir rol oynar. Yüksek oranda kendine tozlaşması ve sınırlı genetik havuza sahip olmasından dolayı varyasyon oldukça düşüktür. Daha önce varyasyon yaratmak amacıyla yapılan doku kültürü çalışmalarda bakla bitkisinin doku kültürü çalışmalarına karşı stabil olmadığı ve inatçı bir bitki olduğu da tespit edilmiştir. Bu nedenle, mikro çoğaltım problemlerinin üstesinden gelmek için, varyasyonda artış sağlayabilecek ve tekrarlanabilir bir mikro çoğaltma protokolünün oluşturulmasına ihtiyaç duyulmaktadır. Aynı zamanda bu sistem verimli gen aktarımı yapabilmeli ve doğrudan organogenez yoluyla geleneksel yetiştirme programlarıyla da entegre edilebilmelidir. Çalışmada, Türkiye'de yetiştirilen iki önemli bakla çeşidi; Filiz99 ve Eresen87 kullanılmıştır. Doku kültürü (sürgün ve köklendirme) protokolü geliştirmek amacıyla 0.05, 0.15, 0.25, 0.35, 0.45 ve 0.55 mg l^{-1} TDZ içeren MS besi yeri embriyonik eksen eksplantı için hazırlanmıştır. Çalışmada Filiz99 ve Eresen87 çeşitlerinde 0.15 mg l^{-1} TDZ içeren MS

ortamında eksplant başına maksimum sürgün sayısı sırasıyla 5.33 ve 3.00 adet elde edilmiştir. Gelişmekte olan sürgünler, üç hafta sonra 1.00 mg l^{-1} IAA içeren MS ortamında köklendirilmiştir. Köklenen bitkileri dış ortama alıştırmak için kontrollü sera koşulları altında torf içeren saksılara aktarılmıştır. Adaptasyon sağlayan bitkilerden tohum elde edilmiştir. Mevcut sonuçlara göre bakla bitkisinde yüksek mikro çoğaltım elde edebilmek için, bitki eksplantının işleme alınmasından önce tohumlar hydropriming tabi tutulması gerektiği ve en uygun yetiştirme ortamının ise 0.15 mg l^{-1} TDZ içeren MS ortamı olduğu sonucuna varılmıştır.

Anahtar kelimeler: Bakla, hidropriming, rejenerasyon, köklendirme, Vicia faba

Introduction

Legumes with high nutritional value make third largest group of plants among all dicotyledonous plant species (Shimaa at al., 2008; Srinath et al., 2005). The faba bean (*Vicia faba* L.) is an important member of this group and is highly nutritional in terms of protein contents. It is very popularly used for food and feed in many parts of the world. There is rapid increase in world population and if proper measures are not taken, there will be a problem to feed growing world population. There is need to improve and breed new cultivars. Both through conventional and biotechnological approaches genetic improvement of faba bean against different types of biotic and abiotic stresses is needed. Biotechnological approaches are helpful for fast and easy incorporation of new traits into plants (Takahoshni and Takomizo, 2012) against different types of biotic and abiotic stresses and improvement for various nutritional characteristics (Shimaa at al., 2008).

Legumes are an excellent source of protein that can help to improve malnutrition among wide range of people around the world. Broad bean has high potential for use as energy and functional food. It is well established under temperate climates with high yield potential (Christou, 1994).
Broad bean is very important edible legume used in number of countries and face number of problems like self-incompatibility with poor genetic diversity that hinders easy development of new varieties (Bond, 1987; Selva et al, 1989). Although, there are number of reports about regeneration from broad bean (Abdelwahd et al, 2008; Abdelwahd et al, 2014; Almaghrabi, 2014; Edyta et al, 2012; Klenotičova et al, 2013; Metry et al, 2007) but all of these studies suggest number of bottlenecks that hinder multiplication of broad bean. There is a lot of scope for improvement of micropropagation. There is need to develop new propagation techniques for its multiplication under *in vitro* conditions for advancement in plant transformation activities.

Therefore, the study aimed to design, optimize and establish new reliable and efficient conditions for mature zygotic embryos based *in vitro* propagation of broad bean.

Materials and Methods

Seed Source

The seeds of broad bean cv. Eresen 87 and Filiz 99 were obtained from the Aegean Field Crops Research Institute, Izmir, Turkey.

Surface Sterilization

Care was taken to select healthy and clean seeds before surface sterilization using 60% commercial bleach (Ace® Istanbul, Turkey, containing 5% NaOCl) for 20 min. The surface sterilized seeds were rinsed 3×5 min with sterile distilled water Thereafter, they were divided in to two sets; one set was hydroprimed in sterile water for 24 h at 24°C. The other set was not hydroprimed. Both hydroprimed and non-primed seed embryos were cultured on MS medium containing 0.05, 0.15, 0.25, 0.35, 0.45, 0.55 mg l^{-1} TDZ (six concentrations) excluding MS medium that served as control. All cultures were autoclaved for 20 min at 121°C, using 1.4 kg/cm^2 autoclave pressure. The pH of all culture media was adjusted to 5.6 - 5.8 with 1M Na OH or1M HCl. All cultures were incubated at 25 ± 2°C under 16 h light (45µmol m^{-2} s^{-1}) photoperiod. Light using cool white fluorescent lamps. The agar, plant hormones and the chemicals that were used in this study were purchased from Duchefa Biochemie B.V., Haarlem, The Netherlands.

In vitro rooting

Healthy and well developed elongated ~ 3 cm long broad bean shoots were rooted on MS medium containing 1.0 mg l^{-1} IAA. All cultures were incubated at 25 ± 2°C in 16 h light/8 h dark cycle in growth chamber. Sixty (60) micropropagated shoots were rooted in each treatment that was divided into 6 replications. Each replication contained 5 micropropagated shoots (5 micropropagated shoots × 12 replications = 60 micropropagated shoots). Data on frequencies of root induction, number of roots per plant, root length, number of shoot per explant if any and flower induction were recorded after three weeks of culture.

***Ex vitro* acclimatization**

In vitro grown well developed healthy ~ 8 cm long plantlets were washed in running tap water to remove adhered agar from roots. Thereafter, the plantlets were transferred to soil mix containing (a) clay loam soil, (b) peat moss, (c) perlite and (d) clay loam soil: peat moss (1:1) soil mix. The clay loam soil had 42% (w/w) clay, 30.00% (w/w) clay and 28% (w/w) sand with 49% water saturation percentage, CEC of 31 cmol/kg, EC 1.25 dS m^{-1}, 0.05% (w/w) total salts, pH of 7.8, 5.14% (w/w) lime, 138.4 kg ha^{-1} phosphorus, potassium of 1744.4 kg ha^{-1}, organic matter of 1.01% (w/w), total nitrogen of 0.09% and organic carbon of 0.81%. Peat moss used in the study was prepared locally from leaves had pH of 6.6 and EC of 0.15 dS/m and porosity of 65% v/w that allowed low bulk density of 0.01 mg m^{-3} and high water absorption. The perlite used in the experiment had a bulk density of about 53 kg/m^3 and contained 71% SiO_2 (w/w), 11% Al_2O_3 (w/w), 4% Na_2O, 2% K_2O, 0.5% Fe_2O_3, 0.2% MgO, 0.5% CaO and 4% loss on ignition (chemical/combined water). The plants were transferred to 1.5 liter plastic pots containing 1.25 liter soil and peat moss mix that were covered with transparent polythene bags. Each pot was given 10 ml water after every two days. During second week of culture; transparent polythene bags were gradually removed after the plants began to show signs of growth. They were watered on weekly basis depending on plants' conditions. Greenhouse used in the experiment maintained temperature of 18 ± 1C and 70 ± 2% relative humidity under 16 h light photoperiod.

Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA), and the post hoc tests were performed using Duncan's Multiple Range test at 0.05 level of significance. The treatments were arranged in a completely randomized design.

Results

The mature embryos are embedded in between very hard and large cotyledon leaves that are difficult to detach to reach them. Therefore, the seeds were hydroprimed for 20 h in autoclaved bi distilled water after carrying out surface sterilization with 60% commercial bleach (Ace – Turkey containing 5% NaOCl) for 20 min with continuous stirring. Hydropriming helped to soften the seeds, which were opened after gentle removal of testa using sharp scalpel blades. All care was taken to detach zygotic embryos without damaging them.

Firstly shoot regeneration percentage of the zygotic embryos was compared on control (MS medium) and MS medium containing 0.05, 0.15, 0.25, 0.35, 0.45 and 0.55 mg l^{-1} TDZ any concentration of TDZ in MS medium failed to induce any callus. Any zygotic embryos on MS medium did not regenerate except one or two developing protrusions on surface of explants after first week of culture that remained static thereafter. Explants began to swell with induction of shoot initials within 7 to 9 days of culture on variable concentrations of TDZ followed by start of shoot regeneration gradually. All hydroprimed mature embryos on cv. Filiz 99 and Eresen 89 induced 100% shoot regeneration (data not shown), with root necrosis noted on some explants after 6 weeks of culture (Figure 1a.). De novo direct shoot regeneration was noted on all zygotic embryos at cotyledon nodes irrespective of the concentration of TDZ. However, they showed significant variation among concentrations for number of shoots per explant and shoot length. This variation was also very obvious for the two characters when the regeneration on two cultivars was compared. The results for number of shoots per explant and their shoot length are described below.

Number of shoots per explant

Number of shoots per explant on cv. Filiz 99 ranged 1.8 to 5.3 (Table1). Maximum number of shoots per explant was noted on MS medium containing 0.15 mg l^{-1} TDZ with variable development of necrosis on some explants. Except 0.15 mg l^{-1} TDZ (with 5.3 shoots per explant) and 0.55 mg l^{-1} TDZ (with 4.3 shoots per explant – Figure 1b.); rest of the cultures induced statistically similar number of shoots per explant. Except one concentration of TDZ (0.45 mg l^{-1}) all concentrations of TDZ induced more number of shoots per explant compared to control (MS medium) treatment.

Number of shoots per explant ranged 2.0 to 3.3 on cv. Eresen 87. Like cv. Filiz 99 (Table1), maximum number of 3.33 shoots per explant was noted on cv. Eresen 87 on MS medium containing 0.15 mg l^{-1} TDZ (Figure 2.). However, low number of regenerated shoots were noted on zygotic embryo of cv. Eresen 87 on TDZ concentrations compared to cv. Filiz 99. Variable necrosis was also noted on cultures of cv. Eresen 87. It seemed all concentrations of TDZ were inhibitory to induce shoots.

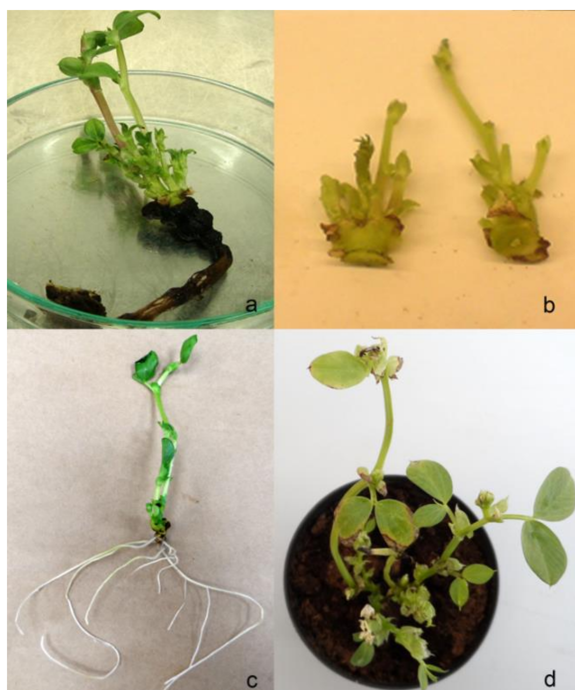

Figure 1. Shoot regeneration on zygotic embryo explant of cv Eresen 87 and Filiz 99. Shoots obtained from zygotic embryo explants (a) Removal of the regenerated prolonged shoots for rooting on black charcoal containing medium (b) Rooting of Filiz 99 shoots on MS medium containing 1 mg l-1 IAA (c) Rooted plants transferred to pots and flowering under greenhouse conditions (d).

As shoots regenerated on any concentration of TDZ did not surpass the number of shoots regenerated on MS medium (control). It was also obvious that all developing shoots on cv. Eresen 87 were more prone to necrosis compared to those developed on cv. Filiz 99. Visible necrosis was prominent on leaves of developing shoots. It is assumed that oxidative stress due to phenolic compounds resulted in deleterious effects on cell walls causing oxidative burst with increased ROS activity leading to death of cells in broad bean in non-hydro primed seeds leading to necrosis.

Shoot length

Shoot length per explant ranged 1.88 to 4.05 cm on cv. Filiz 99 and 2.7 – 10.03 cm on cv. Eresen 87 (Table1). Maximum shoot length per explant for cv. Filiz 99 was 4.05 cm on MS medium containing 0.05 mg l^{-1} TDZ (Figure 3.). Minimum shoot length per explant for cv. Filiz 99 was noted on MS medium (control).

Shoot length on cv. Eresen 87 decreased consistently. Shoot length per explant ranged 2.7 – 10.03 cm on cv. Eresen 87 (Figure 1b.). Maximum shoot length was noted on MS medium containing 0.05 mg l^{-1} TDZ. Each increasing concentration of TDZ in regeneration medium was associated with reduced shoot length. Minimum shoot length was noted on MS medium (control treatment).

Well developed shoots were rooted on MS medium containing 1 mg l^{-1} IAA in plastic pots and acclimatised. These flowered and set fertile seeds (Figure 1c,d.).

Table 1. Effects of various concentrations of TDZ on shoot regeneration from hydroprimed mature zygotic embryos of two broad bean cultivars Filiz 99 and Eresen 87.

TDZ (mg l^{-1})	Filiz 99				Eresen 87			
	Number of shoots per explant		Shoot length (cm)		Number of shoots per explant		Shoot length (cm)	
Control	2.90	bcd	1.80	e	3.00	bcd	4.30	bcde
0.05	3.10	bcd	4.00	cde	2.30	cd	10.00	a
0.15	5.30	a	2.60	de	3.30	bc	8.70	ab
0.25	3.30	bcd	3.20	de	2.60	cd	8.10	abc
0.35	3.10	bcd	3.30	de	2.00	d	6.70	abcd
0.45	1.80	bcd	2.80	de	2.60	cd	5.20	bcde
0.55	4.30	b	2.50	de	3.00	bcd	2.70	de

All values shown in a single column by a different letter are significantly different using Duncans Mutiple range test at P<0.05

Discussion

This study describes a procedure to directly regenerate shoots from zygotic embryos using 2 broad bean cultivars on different concentrations of TDZ. The results of the study emphasize that hydropriming positively facilitated in detaching

zygotic embryos sandwiched between cotyledons. The results are in agreement with my previous observations (Nofouzi et al., 2019). All explants irrespective of the concentration of TDZ swelled before regeneration. However, concentration of TDZ in the regeneration medium affected regeneration variably. The best shoot regeneration with maximum number of shoots per explant was noted on 0.15 mg l-1 TDZ for both cultivars. The longest shoots were noted on MS medium containing 0.05 mg l-1 TDZ used for regeneration. However, the shoots of cv. Eresen attained more than two fold elongation, when compared to shoots regenerated on cv. Filiz 99. Irrespective of the cultivar. Increased concentration of TDZ had negative effects on shoot length.

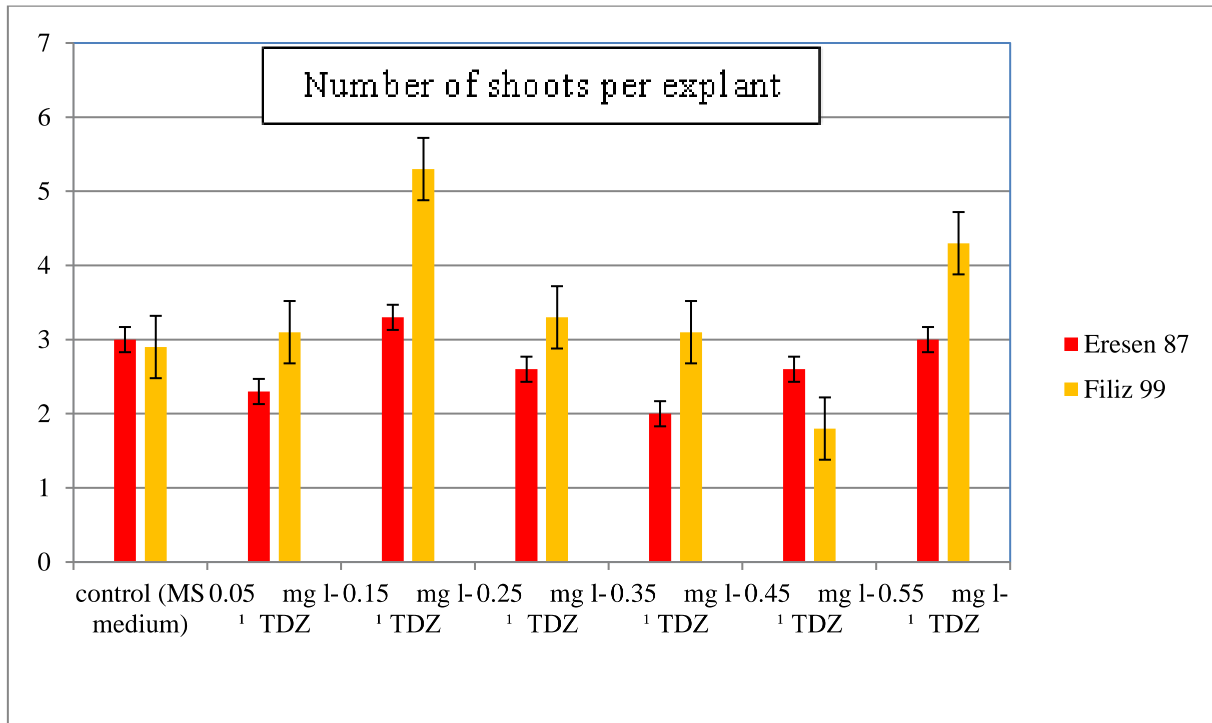

Figure 2. Effects of various concentrations of TDZ on number of shoots per explant of two broad bean cultivars Filiz 99 and Eresen 87

The study aimed to identify suitable strategies for propagation of broad bean under *In vitro* conditions. using different concentrations of TDZ. Previously, micropropagation of broad bean (Abdelwahd et al, 2008; Abdelwahd et al, 2014; Almaghrabi, 2014; Edyta et al, 2012) has been reported with invariable results.

Previous reports suggest that broad bean regeneration and establishment from tissue culture is difficult (Böttinger et al, 2001). Broad bean explants after cutting from the main plant tissues cells undergo oxidation under biotic or abiotic stress due to prevailing flavonoids in the plant like Myricetin, Daidzein, Apigenin and Quercetin, (Almaghrabi, 2014; Anwar, 2007; Anwar et al, 2010; Miean and Mohamed, 2001) that lead to rapid and large generation of ROS radicals in broad bean plant cells. Excessive production of these cells overwhelm natural protection system of plant and lead to cell death (necrosis).

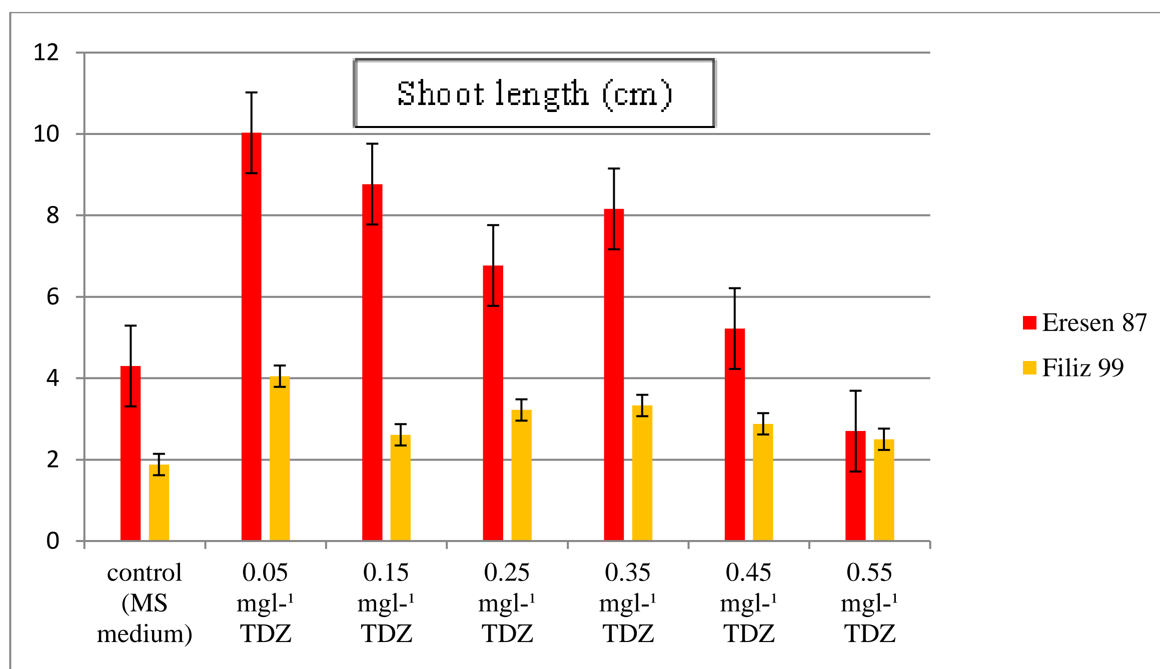

Figure 3. Effects of various concentrations of TDZ on shoot length of two broad bean cultivars Filiz 99 and Eresen 87

Variable necrosis was noted on broad bean regenerates with high intensity on non hydroprimed seeds based explants in this study. This is in agreement with suggested difficulties of indirect regeneration from broad bean due to excessive production of ROS and accumulation of phenolic compounds (Böttinger et al, 2001).

The results of present study suggests that 24 h hydropriming minimize deleterious effects of phenolics and oxidative stress based cell wall burst. Reduced oxidative stress helped in continuous division of cells without hindrance leading to high regeneration percentage (80 - 100%) in this experiment. It was inferred that there is more need to reduce oxidative stress in cells at the start of experiment compared to using antioxidants (Abdelwahd et al, 2008; Metry et al, 2007). This could act as possible remedy to reduce necrosis and improve regeneration.

Except regeneration on few TDZ concentrations, cv. Eresen 87 showed more genetic stability over cv. Filiz 99 in frequency of shoot regeneration percentage.

Direct shoot regeneration was noted on embryonic nodes on MS medium containing all concentrations of TDZ on two cultivars. Maximum number of shoots were noted on cv. Filiz 99. This study has edge over previous studies in terms of time to induce shoots, their rooting and acclimatization that was achieved in 60-65 days. The results are not in agreement with findings from another group (Bahgat et al, 2009). Nofouzi et al. (2019) Obtained the highest mean number of regenerated shoots for Alfalfa was achieved when explants were subjected to 0.55 and 0.15 mg/L TDZ, respectively. They regenerated somatic embryos on two Egyptian broad bean cultivars Giza 2 & Hyto using 10 mg l^{-1} BAP+0.5 mg l^{-1} NAA followed by transfer of the calli to 1/2 B5 medium. Previously a shoot regeneration system has been reported through indirect somatic embryogenesis and organogenesis (Hanafy et al, 2005). Then another group which used shoot tip and epicotyl explants and found possibility of development of embryos over a period of 14 months after number of subcultures (Bahgat et al, 2009). It is assumed that high concentration of BAP used in the study may have caused stress on the explants that led to induction of somatic embryogenesis.

The results of this study reports good shoot length of 1.8 to 4.0 cm on cv. Filiz 99 and 2.7 – 10.0 cm on cv. Eresen 87 that has not been reported in previous studies (Bahgat et al, 2009; Böttinger et al, 2001; Taha & Francis, 1990).

In another study Used primary root with triarch, tetrarch or pentarch vascular bundles of faba bean that play an important role in induction of roots on faba beans (Fahn, 1977). Adventitious roots develop from unusual points of origin as they most commonly arise out of stems, originating via cell divisions of the stem cortex. Adventitious rooting is a unique, complex and essential process in plant propagation (Ford et al. 2001). Rooting is very difficult in faba bean that can be counted as restricting reason to successfully acclimatize regenerated plantlets outside the laboratory

conditions (Metry et al, 2007) To overcome this problem micrografting was done by (Hanafy et al, 2005; Metry et al, 2007). Adventitous root production in higher plants is often affected by thee presence of appropriate root growing auxins plants (Fukaki & Tasaka, 2001).

Roba et al. (2011) Was successful in *In vitro* rooting of four Egyptian cultivars, i.e., Giza 461, Giza 40, Giza 834 and Giza 716 on hormone free MS medium supplemented with 5 mg l^{-1} silver nitrate with maximum rooting on Giza 461 and Giza 40 whereas poor rooting was noted on cv Giza716 and Giza843. The results of this study are not iin agreement to any of the study described above. This study reports 100% rooting under *In vitro* conditions. Most probable reason of differential rooting pattern on all these cultivars could be their internal hormone balance that could vary in all faba bean genotypes.

To identify strategies for successful acclimatization of these plants these were grown on different types of soil mix. Broad bean grows well in soils with pH of 7.0 - 7.5. Clay loam soil used in the study had pH that was a bit higher than that was recommended. Poor organic matter combined with other soil properties also promoted factors that hindered growth and development of roots of tissue cultured plants that are very prone to external environmental conditions.

Ph of peat moss used in the study was lower than appropriate for growth and development of broad bean. Peat moss also had high porosity with high absorption capacity. All these factors may have negatively affected growth and development of roots and hence acclimatization.

Perlite has ability to absorb and accumulate large amount of water. Perlite has ability to hold 200 to 600 percent of its weight in water. Broad bean is a plant that lives on moderate amount of water throughout its life cycle. It is assumed that roots of broad bean plants grown in perlite may have died due to root cell oxidation in water saturated soils even though care was taken not to over flood the substrate.

A mixture of perlite and clay loam may have resulted in development of a soil that was hard with large amount of water that lead to death of roots; hence acclimatization.

A mixture of clay loam soil and peat moss helped in induction of a pH suitable for growth and development of plants' roots in conjunction with surrounding temperature and moderate humidity that helped the plants to grow flourish and acclimatize easily.

Conclusion

The results of this study suggest that broad bean could be micropropagated successfully if the seeds are hydroprimed for 24 hours before taking explants. Positive effects of hydropriming are directly reflected on easy and large number of shoots independent of season. Moreover, the results are improvement over previous studies and these results could have large scale applications in breeding, transformation and functional genomic studies.

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