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GENOTYPIC DIFFERENCES IN THE *IN VITRO* ACCOMPLISHMENT OF *VICIA FABA* LIFE CYCLE

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Abstract: A protocol for *in vitro* regeneration, flowering, fruiting, and production of *in vitro* viable seeds from four *Vicia faba* varieties (Giza-402, Giza-674, Triple-white and Reina blanca) was developed. Callus, multiple shoots and roots were induced to form after eight weeks on three tested nutrient media. However, the efficiency differed with both medium composition and genotypes. Shoots were regenerated either adventitiously on the superficial layers of the nodal stem callus or directly from pre-existing meristems. Highly significant differences among the genotypes were found for the percentage of explants produced callus, % of explants produced shoots, number of shoots per explant, % of rooted shoots and % of flowered shoots. Flower buds occurred on about 12 weeks old shoots. Whether rooted or not, shoots were flowered. Although the *in vitro* flowers and floral organs were smaller in their size than those produced *in vivo* they were perfect and possessed fully developed pistils and stamens. Most of the shoots carried up to 5 bisexual flowers but invariably one pod per shoot with only a single seed was resulted from *in vitro* self-fertilization. The four genotypes significantly exhibited variable flowering performance. The variety Giza-674 showed the highest rate of flowering on the three medium formulations. The embryos were aseptically isolated from the seeds and germinated *in vitro* on the germination medium and the germination rate was found to be 61.4%. The establishment of such novel technique for *in vitro* flowering in faba bean may provide an ideal system for obtaining haploids, gene transfer utilizing microprojectile bombardment, and for rapid breeding of distant genotypes.

Introduction

Broad bean (*Vicia faba* L.) is an important traditional crop used for direct human consumption. Increasing effort is being devoted to research directed towards the improvement of faba bean. One of the major problems with *Vicia faba* is its inability to cross with other *Vicia* species, limiting broadening of germplasm for breeding and development of improved varieties (Albrcht and Kohlenbach 1989). The development of biotechnology for *Vicia* improvement provides the realistic hope for improving *Vicia faba* and expanding its narrow germplasm base (Christou 1992).

Grain legumes have not been particularly amenable to *in vitro*

culture, with plant regeneration from callus proving to be especially difficult. Major problems have been encountered in this connection with large seeded legumes (Dale, 1983) and among these *Vicia faba* has been particularly recalcitrant. In culture, *Vicia faba* tissues often release a substantial amount of phenolic substances and the callus tissue is often characterized by poor growth rates, with tendency to become necrotic after some time (Grant and Fuller, 1968; Schulze *et al*., (1985). Although callus may be initiated, difficulties have been experienced in maintaining the growth of the callus on subculture. Recently, however some progress has been made towards the recovery of shoots from cultured *Vicia faba* tissues. Galzy and Hamoui (1981) obtained shoots from cultured shoot apices and hypocotyls and in the absence of evidence to the contrary these shoots are assumed to be derived from already existing meristems. Shoot development were reported from explants containing axillary meristems by Schulze *et al*. (1985), and Busse (1986), and in addition they obtained shoot regeneration from callus derived from these explants. Fakhrai *et al*. (1989) provided evidence that shoot regenerated from callus initiated from nodal tissue can be adventitious in origin.

Khalafalla and Hattori (2000) improved a new approach to increase the rooting efficiency of regenerated shoots of faba bean by the application of ethylene inhibitors at the appropriate concentrations.

Studies of *de novo* flower formation can be used to investigate flower morphogenesis and may give insight into the morphological, physiological, biochemical and genetic changes that occur during the switch from the vegetative to the reproductive state (Compton and Veilleux, 1991).

In vitro flowering has been reported in many plant species for instance, *Japanese pear* (Tsujikawa *et al*., 1990), *Boronia megastigma* (Roberts *et al*., 1993), switchgrass (Alexandrova *et al*., 1996), Bamboo (Joshi and Nadgouda, 1997), bitter melon (Wang *et al*. 2001), and in tomato (Dielen *et al*. 2001) etc. Conversely, few reports are available in legumes e.g. soybean (Julian and Wyndaele, 1992), *Vigna mungo* (Ignacimuthu *et al*., 1997).

The production of flowers has been possible by manipulating the auxin-cytokinin ratio. Auxin concentrations ranging from 0.1 to 2.2 µM combined with cytokinin (0.1-1 · µM) stimulating the flower bud formation on elongated shoots derived from thin layer explants of *Nicotiana plumbaginifolia* (Kamate *et al*. 1981), *Petunia hybrida* (Mulin and Tran, 1989) and tomato (Compton and Veilleux, 1991).

In 2000 Franklin *et al* studied several factors affecting *in vitro* flowering and fruiting of one green pea (*Pisum sativum* L.) cultivar (PID). The regenerated shoots were rooted and flowered *in vitro* on the 7th and 15th day on rooting medium. The flowers self-fertilized *in vitro* and produced mature pods within 25 days of rooting. These *in vitro* seeds were germinable both *in vitro* and *in vivo*.

The establishment of adequate technique for *in vitro* flowering may provide an ideal system for obtaining haploids, gene transfer utilizing microprojectile bombardment, and for rapid breeding by the employment of the potential use of the mature florets for *in vitro* fertilization techniques and the production of hybrids between genera, species or ecotypes that are difficult to cross normally (Alexandrova *et al.*, 1996, and Franklin *et al.*, 2000).

The objectives of this study were to develop a protocol for *in vitro* regeneration, flowering, fruiting, and production of *in vitro* viable seeds from some *Vicia faba* genotypes.

Materials and Methods

Seeds of four varieties of faba bean (*Vicia faba* L.) namely: Giza-402, Giza-674, and Triple-white (equina) and Reina blanca (major) were surface-sterilized by rinsing in 70% alcohol for 1 min followed by immersion in 20% (v/v) commercial bleach (which contained 5.5% NaOCl) for 30 min. They were then washed four times in sterile distilled water then germinated in 125 ml glass screw capped jars containing 25 ml of nutrient germination medium having the following composition: $Ca(NO_3).4H_2O$, 1.881g l^{-1}; KNO_3, 0.506 g l^{-1}; KH_2PO_4, 0.136 g l^{-1}; $MgSO_4.7H_2O$, 0.244 g l^{-1}; Fe-EDTA, 0.005 g l^{-1}; agar 7.0 g l^{-1} (Roper, 1979). The pH was adjusted to 5.8. Jars were kept in dark at 27 °C. After 2 weeks, the nodal stem explants (0.5-1.0 cm) were excised for culture. The following culture media formulations (based on Murashige and Skoog, 1962) were used:

Medium	Formulation
VF	MS salts +B5 vitamins + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} NAA + 3.0% sucrose.
VFM	MS + 4.0 mg l^{-1} BA+ 5.0gm l^{-1} activated charcoal + 3.0% sucrose.
GF2	Half strength MS macro salts (without nitrate) + MS micro salts + B5 vitamins + 950 mg l^{-1} KNO_3 + 825 mg l^{-1} NH_4NO_3+ 2.0 mg l^{-1} BA + 2.0 mg l^{-1} NAA+ 100 mg l^{-1} Inositol + 2.0% sucrose + 10.0 gm l^{-1} activated charcoal.

Where: NAA = α-naphthyleneacetic acid. BA = 6-benzyladinine.
B5 vitamins after Gamborg *et al.* (1968).

All media were solidified with 0.8% agar (pH was 5.8) and autoclaved for 15 min at 1.2 Kg/cm^{-2}. Explants were placed on the surface of 20 ml of nutrient medium in125 ml screw capped glass jars.

Cultures were incubated in darkness at 15°C for four weeks; subsequently the explants were subcultured onto the same media and maintained at 22°C in a 16 h photoperiod derived from cool white fluorescent tube for another four weeks. The following characters were recorded: % of explants producing callus (Callus %), % of explants regenerating shoots (Regeneration rate), number of shoots per explant (Shoot No.), and % of rooted shoots (Rooting %).

For the induction of *in vitro* flowering the excised regenerated shoots 3-5 cm tall were subcultuerd onto the same media and incubated in continuous illumination at 15°C. After four weeks, the flowers was recoded and the cultures were kept under the same conditions for pod development.

For cytological analysis root tips of regenerated plantlets were removed aseptically and fixed in a 3:1 alcohol-acetic acid mixture. Squash preparations were made using acetocarmine method.

Results and Discussion

Shoot and root regeneration

Callus, multiple shoots and roots were induced, to form after eight weeks (two subcultures) on the tested nutrient media. However, the efficiency differed with respect to both medium composition and genotypes. Shoots were regenerated either adventitiously on the superficial layers of the nodal stem callus or directly from pre-existing meristems (Fig. 1a,b). The means of percentage of explants producing callus, % of explants producing shoots (Regeneration rate), number of shoots per explant (Shoot No.), percentage of rooted shoots (Rooting %), and percentage of flowered shoots (Flowering %) for the four faba bean genotypes are shown in Table (1).

The analysis of variance (Table 2) reveled the presence of highly significant differences among the genotypes for the studied characters. For instance, the variety Triple-white exhibited the highest % of callus formation on both VF and GF2 media (69.1 and 9.5%, respectively) with the highest percentage of rooting (83.2%) on GF2 medium. While, the variety Giza-674 showed high regeneration rate on GF2 medium (87.8%), and high regeneration rate (82.9%) accompanied by high % of rooting (92.2%) on the VFM medium (Table 1). Similarly, the frequency of responding explants varied also among different genotypes of several plants such as tomato

Table (1): The means of percentage of explants produced callus (Callus %), % of explants produced shoots (Regeneration rate), number of shoots per explant (Shoot No.), percentage of rooted shoots (Rooting %), and percentage of flowered shoots (Flowering %) for the four faba bean genotypes Giza402, Triple white, Reina blanca and Giza674.

Genotypes	VF					GF2					VFM				
	Callus %	Regeneration rate	Shoot No.	Rooting %	Flowering%	Callus %	Regeneration rate	Shoot No.	Rooting %	Flowering%	Callus %	Regeneration rate	Shoot No.	Rooting %	Flowering%
za402	63.8	38.2	1.5	0.5	0.5	0.6	62.5	1.6	65.5	0.5	0.3	63.0	1.6	85.1	0.4
white	69.1	58.2	3.1	0.3	0.5	9.5	53.7	2.7	83.2	18.9	0.4	65.2	3.1	77.2	12.3
blanca	60.0	66.0	5.4	0.4	15.0	0.2	80.4	5.8	50.0	21.7	0.6	65.0	5.3	56.5	0.2
za674	35.7	57.1	4.0	0.5	29.2	4.9	87.8	4.7	65.8	19.2	8.6	82.9	3.8	92.2	7.3
EAN	57.15	54.88	3.50	0.43	11.3	3.55	71.1	3.7	66.13	15.08	2.48	69.03	3.45	77.75	5.05

	Callus%	Regeneration rate	Shoot No.	Rooting%	Flowering%
LSD 0.05	3.13	4.86	0.65	3.97	2.60
LSD 0.01	4.26	6.62	0.89	5.41	3.54

Table (2): The analyses of variance for percentage of explants produced callus (Callus %), % of explants produced shoots (Regeneration rate), number of shoots per explant (Shoot No.), % of rooted shoots (Rooting %), and % of flowered shoots (Flowering %) for four faba bean genotypes.

Source	DF	Callus % MS	Regeneration rate MS	Shoot No. MS	Rooting % MS	Flowering % MS
Reps.	2	0.164	1.34	0.06	8.25	0.60
Genotypes (G)	3	150.95**	880.28**	25.27**	669.36**	506.154**
Media (M)	2	11665.60**	935.77**	0.29	21118.07**	307.71**
G X M	6	305.26**	269.54**	0.25	345.67**	225.09**
Error	22	3.429	8.26	0.15	5.52	2.36

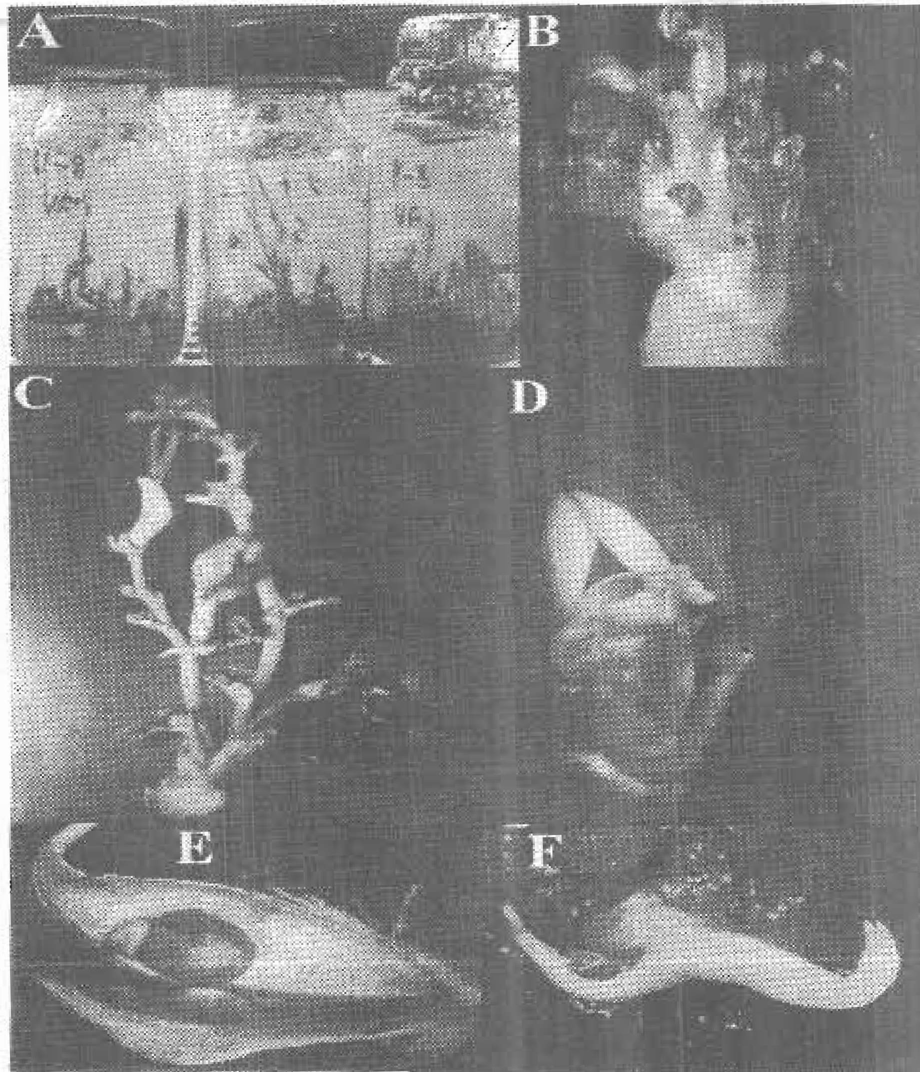

Fig. (1): *In Vitro* accomplishment of *Vicia faba* Life Cycle: (A and B) Shoots regenerated either adventitiously on the superficial layers of the nodal stem callus or directly from pre-existing meristems. (C) Regenerated shoots possessed flower and pod. (D) Perfect flower possessed fully developed pistils and stamens. (E): Mature pod containing single viable seed. (F): *In vitro* germination of embryo isolated from *in vitro* formed seed.

(Compton and Veilleux 1991 and El-Farash *et al.*, 1993) and alfalfa (Fuentes *et al* (1993).

The effects of the different nutrient formulations of the medium were also highly significant for all the studied characters except the number of shoots per explant was not significant (Table 2). For example, the medium VF produced the highest % of callus formation averaged over the four genotypes. Meanwhile, both GF2 and VFM media were superior in the regeneration rate and % of rooting in all the tested varieties (Table 1). There are a number of reports concerning varying nutritional (salts, organic additives, growth regulators, etc....) requirements for *in vitro* clonal propagation of grain legumes such as *Phaseolus* (Rubluo and Kartha, 1985), *Glycine* (Grant, 1984), *Pisum* (Griga *et al.* 1987), and *Vicia faba* (Fakhria *et al.* 1989).

In vitro flowering

Flower buds occurred on about 12 weeks old shoots. Whether rooted or not rooted shoots were flowered (Fig. 1c). This observation was in contrary to Franklin *et al.*, (2000) and Ingnacimuthu *et al.* (1997) who observed that the induction of roots is an important prerequisite for *in vitro* flowering of *Pisum sativum* and *Vigna mungo*. However, flowers could be induced only in the absence of roots in bamboo (Joshi and Nadgauda, 1997).

Although the *in vitro* flowers and floral organs were smaller in their size than those produced *in vivo* they were perfect and possessed fully developed pistils and stamens (Figs. 1d). This observation coincides with the earlier report in *Lycopersicon esculentum* (Rastogi and Sawhney, 1986) however, the *in vitro* flower and floral organs in *Pisum sativum* were more or less similar in size with flowers produced *in vivo* (Franklin *et al.*, 2000). Pierik (1991) found that the floral characters of *in vitro*-produced rose plants were similar to those of plants raised from cuttings. Most of the shoots carried up to 5 bisexual flowers but invariably one pod per shoot with only a single seed was resulted from *in vitro* self-fertilization (Figs. 1c,e). Similar observations were reported for *Vigna mungo* and *Pisum sativum* by Ignacimuthu *et al.*, (1997) and Franklin *et al.*, (2000).

In the present work, because the smaller size of the *in vitro* formed seeds (Fig. 1e), the embryos could be aseptically isolated from the seeds and germinated *in vitro* on the germination medium (Fig. 1f) and the germination rate was found to be 61.4%. Similarly, the *in vitro* formed seeds of *Vigna mungo* and *Pisum sativum* were viable and the *in vivo* germination rate in *Pisum sativum* ranged from 5 to 65% according to the applied auxin (Ignacimuthu *et al.*, 1997 and Franklin *et al.*, 2000).

The four genotypes significantly exhibited variable flowering performance (Tables 1 and 2). The lowest responded variety on the three media was Giza-402 (Table 1). The variety Triple-white flowered on two media GF2 and VFM while, the variety Reina blanca flowered on the two media GF2 and VF (Table 1). Meanwhile, the fourth variety Giza-674 flowered variably on the three medium formulations (29.2, 19.2, and 7.3% of regenerated shoots flowered on VF, GF2, and VFM respectively, Table 1). The significant interactions between genotype and medium (Table 2) confirmed the above observations about the differential flowering performance of the studied genotypes and media formulation.

These results evidently indicate variable *in vitro* requirements of genotypes for all studied characters including *in vitro* flowering, i.e. the variety, which exhibited well performance in some characters on particular medium may perform poorly on another medium. Incidentally, the three types of medium in this investigation did not fit the *in vitro* flowering requirements of the variety Giza-402, so did the medium VF with the variety Triple-white which flowered on the another two media. Similarly, considerable genotypic differences were observed in the expression of *in vitro* flower formation in tomato (Liu and Li 1990). Thus, and considering the numerous factors affecting *in vitro* performance, e.g. type and age of the explants, incubation, plant growth regulators, and medium composition etc. it should be possible to develop various *in vitro* protocols which may match the requirements of wide range of genotypes. This might make it possible, to some extend, to transfer the whole crossing program from the field to the test tube. Franklin *et al.* (2000), reported that if a routine technique and the factors affecting in vitro flowering of pea is established it may provide an ideal system for rapid breeding of distant varieties by synchronization of flowering and hybridizing temperate and tropical varieties to increase the productivity and the value of peas by the introduction of stably inherited traits present in the tropical varieties such as sturdy vines, larger pods, higher yield, early maturity, improved protein quality, herbicide resistance, pests and disease resistance. Furthermore, such versatile system for efficient regeneration, flowering and fruiting of *V. faba* has practical implications for *Agrobacterium*-mediated transformation of *V. faba* plants. There is also a possibility of using it as unique system in the rigorous *in vitro* study of the relationship between *V. faba* and the parasitic plant dodder (*Orbanche* sp.). The adoption of such novel fascinating biotechnologies has the great

potential to aid conventional plant breeding programs.

Chromosomal stability

Chromosomal stability in tissue culture is generally an essential prerequisite for maintenance of genetically defined material to regenerate normal plant from the cultured cells. Most of 12 weeks old root tip cells of regenerated plants showed normal chromosome number with 2n= 12. No diversity of cytological condition and nuclear behavior including anaphasic and telophasic bridge, laggards, rearrangements, and structural changes by breakage and reunion of chromosome are noted. Similar observations were reported in *Vicia faba* by Jha *et al.* (1982). In older cultures (more than 18 weeks) different workers have noted a high variation in chromosome number and structure (Venkateswaran 1963, Jha and Roy 1982).

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الاختلافات الوراثية في قدرة الفول البلدي على إتمام دورة حياته في مزارع الأنسجة

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تم عمل بروتوكول لتكشف نباتات من الفول البلدي حتي وصلت إلى مرحلة الإزهار والإثمار وإنتاج بذور حية باستخدام مزارع الأنسجة وذلك من أربعة أصناف وهي جيزة-٤٠٢ ، جـــيزة-٦٧٤ ، تربل-وايت ، رينابلانكا. تم تكشف كل من الكالوس والأفرع الخضرية والجذور في خلال ثمانية أسابيع على ثلاثة بيئات غذائية مختلفة وقد وجد أن كفاءة الحصول على هذه الكالوســـات تتوقف على كل من التركيب الوراثي ومكونات البيئة الغذائية. وقد حدث تكشف للأفرع الخضرية من الطبقة السطحية للكالوس ومن المرستيم الموجود في النسيج النباتي المنزرع.

وقد وجدت اختلافات عالية المعنوية بين الأصناف المختلفة في قدرتها علي إنتاج الكالوس من النسيج المنزرع و النسبة المئوية لتكوين الأفرع الخضرية وعدد الأفرع الخضرية لكـــل نســيج منزرع وكذا النسبة المئوية للأفرع الخضرية التي أزهرت في مزارع الأنسجة.

تكونت البراعم الزهرية علي الأفرع الخضرية الناتجة من مزارع الأنسجة بعد حوالـــي اثنـــا عشر أسبوعا. وعلي الرغم من أن الأزهار والأعضاء الزهرية المتكونة في مزارع الأنسجة كانت اصغر حجما من المتكونة طبيعيا إلا أنها كانت تحتوي علي الأعضاء المذكرة والمؤنثـــة كاملـــة النمو. هذا وقد كانت الأفرع تحمل عدد من الازهار يصل إلى خمسة أزهار ثنائية الجنس ، ولكنها كانت تحمل قرنا واحدا فقط يحتوي على بذرة واحدة فقط ناتجة من الإخصاب الذاتي. أظـــهرت الأصناف الأربعة معدلات تزهير مختلفة حيث اظهر الصنف جيزة-٦٧٤ اعلي معدلات تزهـــير علي البيئات الغذائية الثلاث التي تم اختبارها. تم عزل الأجنة من البذور المتكونة في الأنبـــوب تحت ظروف التعقيم أمكن إنباتها على البيئة الغذائية الخاصة بالإنبـــات وكـــان معـــدل الإنبـــات ٦١,٤%. وقد اقترح أن تطوير هذه التقنية الحديثة لتذهير وإثمار الفول البلدي في مزارع الأنسجة تمثل نظام نموذجي للحصول علي النباتات الأحادية ونقل الجينات عن طريق الهندسة الوراثيـــة وسرعة إنجاز برامج التربية والتي يمكن إن تستخدم فيها تراكيب وراثية بعيدة القرابة.