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In vitro Micropropagation of (*Vicia faba* L.) Cultivars 'Waza Soramame and Cairo 241' by Nodal Explants Proliferation and Somatic Embryogenesis

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Abstract: *In vitro* micropropagation of *(Vicia faba* L.) cultivars 'Waza Soramame and Cairo 241' has been achieved by somatic embryogenesis and nodal segment explants raised seedlings. The influence of nodal segment explant age, genotype and phytohormones on micropropagation both cultivars has been investigated. The explants were cultured on MS media containing different concentrations and combinations of cytokinin (BPA, TDZ, BA) plus auxin (NAA). Among difference and combinations, BAP + NAA showed the best result and the highest number (83 and 78%) of multiple shoots for 'Waza Soramame and Cairo 241', respectively was observed in MS media containing 2.0 mg L^{-1} BPA + 0.2 mg L^{-1} NAA. Cultivar Waza Soramame was more regenerative than Cairo 241 in terms of shoot per explant. Multiplication was also achieved by use only (0.58 mg L^{-1} TDZ or 1.24 mg L^{-1} BA) in combination with 2.15 mg L^{-1} NAA. Embryogenic tissues were initiated from zygonic embryos cultured on MS medium supplemented with IAA. After transfer of embryogenic tissues with developing embryoids on media lacking IAA and supplemented with low concentration of 2,4-D, the development of somatic embryos was enhanced. Secondary somatic embryogenesis led to the formation of new adventive somatic embryos.

Key words: Micropropagation, somatic embryogenesis, nodal segments, multiple shoot, *Vicia faba*

INTRODUCTION

Several, plant Leguminosaes have been mass propagated by tissue culture technology, mainly seeds and ornamental plants (Malik and Saxena, 1992). While the tissue culture technology has been developed for the mass propagation of several seeds plant species, several other plant Fabaceaes are lagging behind due to their recalcitrant nature to *in vitro* techniques (Polanco and Ruiz, 1997). *Vicia* is a multipurpose plant species widely grown for its seeds, fodder, fuel and medicinal purposes. It belongs to the botanical family Leguminosae (Fabaceae) and is generally propagated by seeds. Several cultivars have been selected among the seedling populations for their superior seed quality. Seed propagation method is generally used since it is homozygous in nature. *Vicia faba* L. cv. Waza soramame is an improved Japanese cultivar multiple propagated by seed methods (Khalafalla and Hattori, 1999). There is also an increased knowledge of the complexity of issue: the inter-relationship development, population growth and agricultural production (Cincotta *et al*., 2000; Livernash and Rodenburg, 1998; Population Reference Bureau, 2002; United Nations Environment Programme, 1992; Vitousek, 1997). Botanists are interested in faba bean development of an efficient *in vitro* propagation because of its potential to multiply economically important genotypes in large cultivars.

Crop yield can be enhanced significantly by large-scale multiplication of selected genotypes with improved growth rates, high stress tolerance and disease resistance. *In vitro* techniques such as micropropagation provide a fast and reliable method for production of large number of uniform plantlet in short time. Direct multiple shoot production from different parts of seedling has been reported in *Albizia lebbek*, *Albizia odorastissima*, *Delonix regia* and some other legume plants. Micropropagation of *Delonix regia* through immature embryo drived shoot tips (Rahman and Hossain, 1992) and micropropagation of *Caesalpinia pulcherrima* through nodal bud culture of mature tree (Rahman *et al*., 1993). The most efficient induction in (*Vicia faba* L.) shoot was the combination of BA and TDZ, shoot rooted in half-streng MS medium or after the addition of IAA (Khalafalla and Hattori, 2000). Some aerial reports used nodal segment as an explant for which stem and stem seedling were used (Prabha *et al*., 2000; Sing and Chaturvedi, 2002; Hassan and Takagi, 1995; Kameswara

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Rao, 2004). Present experiments proved that micropropagation may be a practical solution for a rapid propagation of selected genotypes with desirable properties.

The present study was undertaken to determine the culture conditions for rapid induction, an efficient protocol for *in vitro* plant regeneration from nodal segments *in vitro* grown seedlings and somatic embryogenesis *Vicia faba* cultivars viz., Waza soramame and Cairo 241.

MATERIALS AND METHODS

Seed material: The material seeds of the cultivar *Vicia faba* plants line (Cairo 241) was obtained from Department of Plant Science, National Research Centre, Egypt and the material seeds of *Vicia faba* L. cv. Waza soramame plants was collected from Matsunaga Shubyou Seed Company Ltd., Japan. Healthy and uniform seeds for two cultivars were aggregated and surface sterilized in 70% (v/v) ethanol for 1 min followed by 10 min immersion in 3% (v/v) sodium hypochlorite solution with 2 drops of Tween-20 per 100 mL and then washed 4 times with sterile distilled water. After soaking for 8 h in sterilized distilled water, dried on a sterile paper towel.

For regeneration from nodal segments: Surface sterilized seeds were aseptically transferred singly into each conical flask containing 40 mL of the medium phytohormone - free half - strength MS (Murashige and Skoog, 1962) salts medium of the macro-and micro-nutrients of the Murashige and Skoog and then placed on nutrient medium for germination. Nodal segments of about 1-1.5 cm for each cultivar (*V. faba* L. cv. Waza soramame and *V. faba* L. cv. Cairo 241) were removed from 30 or 60 days old *in vitro*-raised seedlings and used as initial material micropropagation of faba bean using method of shoot bud proliferation. Zygonic embryos were used as initial explants for propagation of faba bean via somatic embryogenesis. Each node was incubated basal medium containing (BPA, 0.5, 1.0, 2.0 and 4.0 mg L^{-1}) in combination with (NAA, 0.1, 2.0 and 4.0 mg L^{-1}), in another treatment which (TDZ, 0.58 mg L^{-1} or BA, 1.24 mg L^{-1}) in combination with NAA 2.15 mg L^{-1} for shoot differentiation. MS salts with glutamine (50 mg L^{-1}) and myo-insitol (100 mg L^{-1}) were used as basal medium for all experiments. The explants were cultured in 5.5×10.5 cm bottle contained 40 mL of nutrient medium. In most cases explants were cultured for four weeks cultured period and then transported to fresh medium for further multiplication. The entire nodales from 30 or 60 days old seedlings were used for initiatings cultures. Ten explants were used for each treatment and all the best treatments were repeated 3 times (total of 30 cultures per treatment). The number of responding culture as well as the number of shoot buds/nodal stem explant per culture were recorded at regular intervals. After four weeks of culture the nodal segment explants, responded to the MS medium with different concentration of BPA in combination with NAA (Table 1) and (BA or TDZ) in combination with NAA (Table 2) and they were transferred to hormone-free medium showed continuous growth and multiplication (Fig. 1d).

In the second set of experiments for induction embryogenic culture: Seeds of each of the cultivars were

Table 1: Response of nodal explants in percentage of 'Waza Soramame and Cairo 241' on Ms medium with different combination of plant regulators. Thirty explants drived from seedling raised in bottles were cultured per treatment

	Explant source							
	Vicia faba L. cv. 'Waza Soramame'				*Vicia faba* L. cv. 'Cairo 241'			
	Nodal age (30 days old)		Nodal age (60 days old)		Nodal age (30 days old)		Nodal age (60 days old)	
Treatments (mg L^{-1}) BAP + NAA	Response (%)	Average No. of shoots/explant (mean±SE)	Response (%)	Average No. of shoots/explant (mean±SE)	Response (%)	Average No. of of shoots/explant (mean±SE)	Response (%)	Average No. of shoots/explant (mean±SE)
0.5 + 0.1	27	1.4±0.84	25	1.6±0.84	29	1.5±0.95	18	1.2±0.40
1.0 + 0.1	40	1.6±0.87	30	1.7±1.01	43	1.6±0.90	35	1.5±0.87
2.0 + 0.1	64	2.2±1.43	55	1.9±1.15	55	1.9±1.10	48	1.8±1.08
4.0 + 0.1	47	2.2±0.86	36	1.9±1.27	50	1.8±0.96	32	1.7±1.17
0.5 + 0.2	43	1.8±1.01	31	1.8±0.95	38	1.8±1.02	30	1.5±0.80
1.0 + 0.2	58	2.0±1.12	49	2.0±1.32	44	1.8±1.14	40	1.7±1.09
2.0 + 0.2	83	3.0±1.09	71	2.4±1.08	78	2.2±1.16	20	1.3±0.64
4.0 + 0.2	53	1.8±0.92	42	2.0±1.01	51	1.9±0.98	36	1.5±0.67
0.5 + 0.4	57	2.0±1.03	41	1.9±1.20	49	1.9±1.03	45	2.0±1.22
1.0 + 0.4	49	1.7±0.91	38	1.7±1.05	45	1.8±1.02	42	1.7±1.03
2.0 + 0.4	74	2.5±1.02	53	2.0±1.21	66	2.5±1.14	48	1.8±1.13
4.0 + 0.4	61	2.1±1.10	46	1.7±0.91	50	1.8±1.21	37	1.5±0.75

Table 2: Effect of cultivars types, node segments and cytokinin types on shoot proliferation of *Vicia faba* L.

	Vicia faba L. cv. 'Waza Soramame'				*Vicia faba* L. cv. 'Cairo 241'			
	Nodal (30 days old)		Nodal (60 days old)		Nodal (30 days old)		Nodal (60 days old)	
Treatments	Response (%)	Shoots/Explant (mean±SE)	Response (%)	Shoots/Explant (mean±SE)	Response (%)	Shoots/Explant (mean±SE)	Response (%)	Shoots/Explant (mean±SE)
BA + NAA	57	2.1 + 1.02	43	1.7 + 1.05	51	1.8 + 1.04	34	1.5 + 0.89
TDZ + NAA	60	2.4 + 1.20	52	2.0 + 1.09	45	1.5 + 1.0	40	1.03 + 0.95

Data based on "Waza Soramame and Cairo 241" nodal segments cultured on modified MS medium supplemented auxin (NAA 2.15 mg L^{-1}) and cytokinin (BA 1.24 mg L^{-1}, TDZ 0.58 mg L^{-1})

previously sterilized above, by the sterilized seeds were rinsed two times with sterile deionized water and after soaking for 8 h in sterilized water , dried on a sterile paper towel. Immature and mature embryos, 2.5 and 3.0 mm long, were aseptically excised with a scalpe from these seeds and were placed onto media containing MS salts and vitamins MS supplemented IAA (0.2, 0.4, 0.8, 1.0 and 2.0 mg L^{-1}) alone or in combination with BA (0.5, 1.0 mg L^{-1}) and contained in plastic Petri dishes (9 cm diameter, 1.5 cm tall). After incubation, the embryogenic cultures with developing empryoids were transferred to MS medium lacking IAA and supplemented with 2,4-D (0.2 and 0.4 mg L^{-1}). The chemicals used in the present study were purchased from *Sigma-Aldrich*. The pH of all the media was adjusted to 5.7 with KOH or HCl before adding the appropriate % (w/v) of agar powder (INA BA-30) and then autoclaved at 121°C for 20 min. All cultures were incubated at 25±2°C with a 16 h light/8 h dark cycle at a light intensity of 25 μ mol $m^{-2}s^{-1}$ and each bottle after inoculation was covered by one layer of polypropylene plastic sheet.

Statistical analysis: All the experiments were repeated three times and the standard deviation was calculated. Data on nodal segment regeneration and somatic embryogenesis were statistically analysed using a completely randomized block design and means were evaluated at the p = 0.05 level of significance using Duncan's New Multiple Range Test.

RESULTS

Effect of growth regulators: The present findings of *V. faba* L. cultivars 'Waza soramame and Cairo 241' demonstrate the possibility for micropropagation of faba bean through nodal and somatic embryogenic cultures. After four weeks of culture the nodal segment explants responded to the MS medium with combinations of various concentrations of cytokinin (BPA) and auxin (NAA) (Table 1). All the nodal segment explants showed callusing at the cut end (Fig. 1a-c) touching the medium in all the treatments. Callus growth and proliferation was 100% in the medium containing (1.24 mg L^{-1} BA, TDZ 0.58 mg L^{-1} in combination with 2.15 mg L^{-1} NAA) (Table 2) and also with different concentration of BPA in combination with NAA (Table 1). The nodal segment explants grown on half-strength MS medium supplemented with 1.24 mg L^{-1} BA or 0.58 TDZ mg L^{-1} in combination with NAA 2.15 mg L^{-1} (here after mentioned as optimal medium) was most effective and shoot regenerated directly from nodal segment explants of both cultivars between 30-45 days of culture. Single nodal explants gave multiple shoot buds (Fig. 1b) after 28 days in the medium with 4.0 mg L^{-1} BPA with 0.2 mg L^{-1} NAA, while in the hormmone-free-medium each node produced a single shoot bud. The use of TDZ or BA higher than (1.24 and 0.58), respectively promoted callus formation. Of all auxins tested, NAA was most effective for direct shoot regeneration, whereas IAA and 2,4-D were preventibly to regeneration but improved callus formation and presence of NAA higher than 2.15 mg L^{-1} in combination with (TDZ or BA) decreased the number of regenerated shoots. The combination and concentration of growth regulators were vital in inducing a suitable regeneration response. The development of multiple shoots from nodal stem explants started after four weeks of culture. Media were initially screened on the basis of the number of explants that responded in term of nodal stem regeneration. Media that showed above 60% response in of Waza soramame and 55% in case of Cairo 241 were considered for further studies (Table 1) at age 30 days for two cultivars, which included 0.2 mg L^{-1} BAP in combination with 0.1, 0.2 and 0.4 mg L^{-1} NAA in case of Waza soramame, also media that showed TDZ in combination with NAA the better than BA in combination with NAA for induction shoot proliferation for two cultivars were considered for studies (Table 2). The shoots kept in the culture medium for longer duration without subculture, showed shedding of leaves and harding of stem. All shoots maintained in cultures for more than two months without subculture failed to produce any adventitious roots when transferred to the rooting medium. One-month-old shoots with 3-4 nodes, when transferred to the rooting medium containing 5 mg L^{-1} IBA, produced adventitious roots after 28 days of culture. It was observed that only 20% of the shoots produced adventitious roots in the rooting medium with 5 mg L^{-1} IBA. All the plants survived during the acclimalization stage.

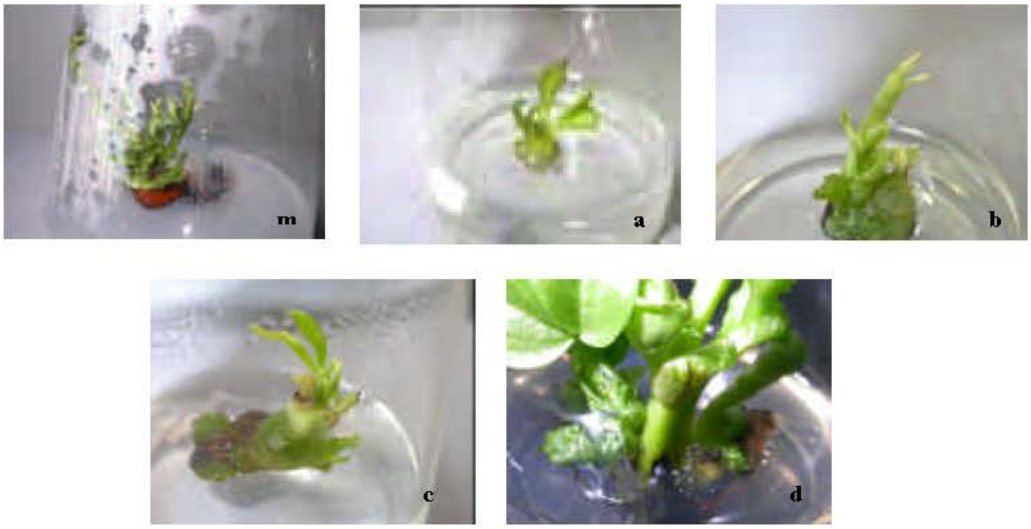

Fig. 1: Different stages of in vitro regeneration of Vicia faba from nodal stem explant. m) Seedling 30-60 days a) allus and shoot bud formation from nodal stem. b) Callus and shoot developing from nodal stem. c and d) rowing shoot of Cairo '241' on MS medium containing 2.0 mg L^{-1} and 0.2 mg L^{-1} NAA

Fig. 2: Secondary somatic embryogenesis and development of new adventive somatic embryos in *Vicia faba* embryogenic culture

Fig. 3: Plantlet of *Vicia faba* regenerated from somatic embryo

Effect of cultivar: The effect of cultivar difference was independently investigated by culturing the young nodal segment explants 30 d of both cultivars on the optimal medium 2.0 mg L^{-1} BAP plus 0.2 mg L^{-1} NAA in Table 1 and 1.24 mg L^{-1} BA or 0.58 mg L^{-1} TDZ in combination with NAA 2.15 mg L^{-1} in Table 2. Though both cultivars, Waza soramame and Cairo 241, exhibit direct shoot induction, the time of shoot initiation, growth rate, and number of shoots varied with the cultivar Waza soramame began to form shoots between 28-35 days, while shoot formation began between 35-40 days for Cairo 241. Sinha *et al.* (1993) observed regeneration of one from six tested genotypes of grass pea (*Lathyrus sativus* L.) from stem segments.

Induction of somatic embryos and plant regeneration: The embryogenic tissue was initiated on modified MS medium supplemented with IAA. Zygotic embryos of two cultivars excised from seeds and produced embryonic cultures frequently. Immature zygotic embryos produced embryogenic tissues during the continuous exposure on medium containing IAA. Embryogenic tissues developed within 4-8 weeks in cultures, and yellowish-green globular structures differentiated on MS medium containing IAA.

The most effective range of IAA concentration was 0.2-1.0 mg L^{-1}. After 6-10 weeks in cultures, globular structures developed, later giving rise to somatic embryos. Secondary somatic embryogenesis was frequent and let to the formation of new adventive somatic embryos. The process of somatic embryo development from the initial globular stage to the bipolar embryo occurred on MS medium containing IAA. However, IAA generally arrested the development of somatic embryos, and only embryoids that were not in direct contact with the medium, exhibited good development. The development of somatic embryos was enhanced after transfer of embryogenic tissues with developing embryoids on MS medium lacking IAA and supplemented with a low concentration of 2,4-D (0.1-0.2 mg L^{-1}). Secondary somatic embryogenesis, the process when new somatic embryos are produced on the surface of previously differentiated somatic embryos, was frequent (Fig. 2). Produced somatic embryos exhibited good capacity for secondary somatic embryogenesis. Secondary somatic embryos were produced on hypocotyls and root regions of the primary somstic embryos. Secondary somatic embryogenesis has been used for multiplication of somatic embryos. *Vicia* embryogenetic cultures have been maintained by subculture of secondary somatic embryos. The germination and conversion of *Vicia faba* L. cultivars 'Waza soramame and Ciro 241' somatic embryos to plants was stimulated on media supporting coordinated development of shoot and roots (Fig. 3). The conversion of somatic embryos to plants was achieved on modified MS media containing a low concentration of 2, 4-D (0.1-0.2 mg L^{-1}) that supported the growth and development of shoot.

DISCUSSION

New biotechnological methods have a great significance for preservation and reproduction of valuable genotypes. The application of biotechnological methods will enable a shortening of time-consuming reproductive processes. Organogenesis and somatic embryogenesis in *Vicia* species was described in past years (Khaiafalla and Hattori, 1999; Kubalakova and Griga, 1989; Lazaridou *et al*., 1993; Mohamed *et al*., 1992). Since that time, significant progress has been achieved. The method of nodal segment explants for micropropagation appears to be a suitable method for *in vitro* fast reproduction of (*Vicia faba* L.) both cultivars. The principal benefits of this method are that the multiplication rates are high. Our protocol is based from nodal segments with high efficiency and is highly suitable for genetic transformation. Further refinement of nodal stem medium on two cultivars is being carried out in our laboratory.

Somatic embryogenesis is believe to be a technique methods of producing great number of plants in a short time. Advances appearance have increased the production of somatic embryos and the frequency of their changeableness to plants. The plant regeneration and the induction of embryogenic is highly used by exogenous phytohormones. The appropriateness of phytohormones can frequently is vital in the induction of somatic embryogenesis. Formation of *Vicia* embryogenic cultures was carried out in the presence of exogenous auxin. In depth insight toward factors effecting somatic embryogenesis can induce to their regulation and improvement of the somatic embryo initiation and conversion to plants. Since the initial report of somatic embryogenesis in *Phaseolus* species (Zambre *et al*., 1998) and *Vicia* species (Lazaridou *et al*., 1993) significant progress has been achieved in *Vicia* propagation by somatic embryogenesis. Auxin was showed to be basic for induction of somatic embryos for the secondary somatic embryogenesis. The secondary embryogenesis is used for proliferation of somatic embryos and embryogenic cultures have been kept by subculturing of secondary embryos.

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REFERENCES

Cincotta, R.P., J. Wisnewski and R. Engelman, 2000. Human population in the biodiversity hostpots. Nature, 404: 990-992.

Hasan, S.M.Z. and H. Takagi, 1995. Alginate-coated nodal segments of yam (*Dioscoria* spp.) for germplasm exchange and distribution. IPGRI/FAO Plant Genet. Resour. Newsl., 103: 32-35.

Kubalakova, M. and M. Griga, 1989. Somatic embryogenesis in *Vicia faba* L. Plant. Tiss. Org. Cult., 9: 167-171.

Kameswara Rao, N., 2004. Plant genetic resources: Advancing conservation and use through biotechnology. African J. Biotechnol., 3: 136-145.

Khalafalla, M.M. and K. Hattori, 1999. Acombination of thidiazuron and benzyladenine promotes multiple shoot production from cotyledonary node explants of faba bean (*Vicia faba* L.) Plant Grow. Regul., 27: 145-148.

Khalafalla, M.M. and K. Hattori, 2000. Differential *in vitro* direct shoot regeneration responses in embryo axis and shoot tip explants of faba bean. Breeding Sci., 50: 117-122.

Lazaridou,T.B., D.G. Roupakias and A.S. Economou, 1993. Embryo rescue in *Vicia faba* and *Vicia narbonesis*. Plant Tiss. Org. Cult., 33: 297-301.

Livernash, R.and E. Rodenburg, 1998. Population change, resources and the environment. Pop. Bull., No. 53.

Malik, K.A. and P.K. Saxena, 1992. Somatic embryogenesis and shoot regeneration from intact seedlings of *Phaseolus acutifolius* A., *P. aureus* (L.) Wilezek., *P. coccineus* L. and *P. wrightii* L. Plant Cell Rep., 11: 163-168.

Mohamed, M.F., P.E. Read and D.P. Coyne, 1992. Dark preconditioning, CPPU and thidiazuron promote shoot organogenesis on seedling node explants of common and faba beans. J. Am. Soc. Hortic. Sci. Jul., 117: 668-672.

Polanco, M.C. and M.L. Ruiz, 1997. Effect of benzylaminopurine on *in vitro* development and *in vivo* root development in lentil, *Lens culinaris* Medik. Plant Cell Rep., 17: 22-26.

Population Reference Bureau, 2002. 2002 World Poulation Data Sheet.

Prabha, B., J. Ila, J.M.S. Chauhan, S.K. Sharma and S.K. Bagchi, 2000. Microprpagation of A 23-year-old candidate plus of *Eucalyptus tereticornis* Sm. Ind. J. For., 23: 149-154.

Rahman, S.M. and M. Hossain, 1992. Micrpropagation in *Delonix regia*. Pak. J. Bot., 24: 60-63.

Rahman, S.M., M. Hossain, B.K. Biswas, O.I. Joarder and R. Islam, 1993. Microprpagation of *Caesalpinia pulcherrima* through nodal bud culture of mature tree. Plant Cell Tissue Organ Culture, 32: 363-365.

Sinha R.R., K. Das and S.K. Sen, 1993. Plant regeneration from stem-derived callus of the seed legume (*Lathyrus sativus* L.) Plant Cell Tissue Org. Cult., 2: 67-76.

Singh, S.K. and H.C. Chaturvedi, 2002. Production of Cloned Plants of *Citrus aurartifolia* (Christm) Swingle and *C. sinensis* (Linn.) Osbeck Through Prolonged *in vitro* Culture of Nodal Stem Setem Segment of Mature Trees (Eds. Nandi S.K., L.M.S. Palni and A. Kumar). Role of Plant Tissue Culture in Biodiversity Conservation and Economic Development. Prakashan, Nainital, pp: 555-564.

United Nations Environment Programme, 1992. Rio Declaration on Envitronment and Development.

Vitousek, P.M., 1997. Human dominantion of earth's ecocystems. Science, 277: 494-499.

Zambre, M.A., J. Clerq and E. Vranova, 1998. Plant regeneration from embryo derived callus in *Phaseolus vulgaris* L. (common bean) and *P. acutifolius* A. Grey (tepary bean). Plant Cell Rep., 17: 626-630.