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The Regeneration of Bean Plants from Meristem Culture

By

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With 4 Figures

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Summary

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We studied the growth and development of *Phaseolus vulgaris* L. cv. Zorin meristems in vitro. Special attention was paid to the influence of different types of cytokinins and their concentration on bud induction, shoot growth and callus formation. The appropriate conditions of seed germination were established in order to separate seeds with various internal seed diseases prior to meristem isolation. Basal media was supplemented with 0.1–10 μmol/l benzyladenine, or with 1–10 μmol/l 6-[γ,γ-dimethylallylamino]-purine, or with combinations of benzyladenine and gibberellic acid or α-naphthalene acetic acid. Although the buds developed on all media used, the regeneration of plants able to survive in soil was achieved only when meristems were isolated on basal medium supplemented with 1 and 5 μmol/l benzyladenine or 6-[γ,γ-dimethylallylamino]-purine and with 20 μmol/l benzyladenine and 1.4 μmol/l gibberellic acid. The most vigorous plants developed when 1 μmol/l 6-[γ,γ-dimethylallylamino]-purine was used for initial meristem culture and was further incorporated in the elongation media. According to these results, we worked out a system for meristem regeneration, elongation and rooting, and prepared the protocol for production of mature bean plants.

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Zusammenfassung

BENEDIČIČ D., RAVNIKAR M. & GOGALA N. 1997. Die Regeneration von Bohnen aus Meristemkulturen. – Phyton (Horn, Austria) 37 (1): 151–160, 4 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Wir untersuchten das Wachstum und die Entwicklung von Meristemen von *Phaseolus vulgaris* L. cv. Zorin in vitro. Besonders wurde auf den Einfluß verschiedener Cytokinine und ihrer Konzentrationen auf die Knospeninduktion, das Sproßwachstum und die Kallusbildung geachtet. Geeignete Bedingungen für die Samenkeimung wurden entwickelt, um Samen mit verschiedenen Krankheiten vor der Isolierung für Meristeme auszuscheiden. Die Nährmedien wurden mit 0,1–10 µmol/l Benzyladenin, mit 1–10 µmol/l 6-[γ,γ-dimethylallylamino]-purin, oder mit Kombinationen von Benzyladenin und Gibberellinsäure oder α-Naphtalenessigsäure versetzt. Obwohl sich Knospen in allen verwendeten Medien entwickelten, gelang eine Regeneration von Pflanzen, die auch in der Erde überleben konnten, nur, wenn die Meristeme auf einem Grundmedium, welches mit 1 und 5 µmol/l Benzyladenin oder 6-[γ,γ-dimethylallylamino]-purin und mit 20 µmol/l Benzyladenin und 1,4 µmol/l Gibberellinsäure versetzt war, isoliert wurden. Die stärkste Pflanzenentwicklung erfolgte, wenn 1 µmol/l 6-[γ,γ-dimethylallylamino]-purin von Anfang an der Gewebekultur und weiterhin auch dem Medium für das Längenwachstum beigefügt wurde. Aufgrund dieser Ergebnisse arbeiteten wir ein System sowohl für Meristemregeneration als auch Sproßwachstum und Wurzelbildung aus und entwickelten eine Vorschrift für die Produktion fertiger Bohnenpflanzen.

Introduction

Several bacterial, fungal, and virus diseases are transmitted with bean seeds (RICHARDSON 1979, SCHUSTER & al. 1987). Meristem culture has proved to be a useful tool in eliminating pathogens from different crop and horticultural plants (QUAK 1977). Although recalcitrant bean meristem cultures have been applied in mass propagation and elimination of viral pathogens including seed-borne viral infections (RUBLUO & KARTHA 1985, SAAM & al. 1987) and germplasm preservation with consequent exchange of genetic material (KARTHA 1982, RUSSELL & al. 1993). According to our knowledge, regeneration from meristems of only rare bean cultivars have resulted in plantlets that grow to maturity: cv. Taylor (RUBIES-AUTONELL & al. 1982) and Dwarf Green Stringless (KARTHA & al. 1981, RUBLUO & KARTHA 1985, GANTOTTI & al. 1985). RUSSELL & al. 1993 reported also the regeneration of transformed navy bean plants.

The successful development of plantlets from meristem depends on changing the media constituents for each stage of development. RUBIES-AUTONELL & al. 1982 cultured meristem tips to produce virus-free plants. The best results were obtained by growing them on MS medium with 20 µmol/l BA and either 3 µmol/l NAA or 1.4 µmol/l GA_3, transplanting derived shoots on to MS without hormones, and 2 weeks later on MS containing 2 mg/l IAA and 3 mg/l IBA. RUBLUO & KARTHA 1985 also reported the need for different hormone combinations for different developmental stages.

Media requirements for bean cultures can vary from cultivar to cultivar (MOK & MOK 1977). RUBLUO & KARTHA 1985 recognised that of three bean cultivars each had different patterns of response in bud and shoot regeneration when cultured on the same medium. Cultivated meristems of individual cultivars need different concentrations and ratios of cytokinins or/and auxins. Furthermore, they observed cultivar specific growth of meristems with different types of cytokinins and auxins. The present study was undertaken to evaluate the ideal conditions and media for the bean cv. Zorin meristem culture. A protocol for regeneration of mature bean cv. Zorin plantlets suitable for routine utilisation was established.

Materials and Methods

Abbreviations: BA = benzyladenine; BA = basal medium; B5 = Gamborgs B5 medium (Gamborg et al., 1968); 2iP = 6-[γ,γ-dimethylallylamino]-purine; GA_3 = Gibberellic acid; IAA = indole-3-acetic acid; IBA = indole-3-butyric acid; NAA = α-naphthalene acetic acid; MS = Murashige and Skoog medium (MURASHIGE & SKOOG 1962); MSB5 = medium consist of MS mineral salts and B5 vitamins.

Seeds of *Phaseolus vulgaris* cv. Zorin were disinfected for 1 min in 70% ethanol and for 20 min in a 1% sodium hypo chlorite on a shaker. Subsequently the seeds were washed three times with sterile distilled water. Seeds that lost their colour after sterilisation treatment, had usually been damaged by various internal diseases and were discarded. Seeds germinated at 25°C in test tubes on water-agar medium (0.6% agar) for 4 days with a 16 h photoperiod, 3 days in complete darkness and then again 4 days with a 16 h photoperiod. In this way all latent bacterial diseases appeared during germination. This procedure was chosen after comparing the germination of seeds in different illumination conditions and in the dark. After 10–12 days of germination, shoot apical and lateral meristems, usually 0.3–0.6 mm in length containing 2–4 leaf primordial, were aseptically isolated from seedlings and cultured.

The basal medium (BM) consisted of mineral salts according to MURASHIGE & SKOOG 1962 modified by BHOJWANI & RAZDAN 1983 as follows: $FeSO_4*7H_2O$ and $FeEDTA*2H_2O$ were replaced with NaFeEDTA (136 µmol) and $ZnSO_4*7H_2O$ was used in a concentration of 36.85 µmol. B5 vitamins (GAMBORG & al. 1968), 3% sucrose and 0.8% Difco Bacto-agar were added.

For meristem growth induction, the basal medium was supplemented with 0.1, 1, 5, 10 µmol/l BA (KARTHA & al. 1981), or with 20 µmol/l BA and 1.4 µmol/l GA_3, or with 20 µmol/l BA and 3 µmol/l NAA (RUBIES-AUTONELL & al. 1982) or with 1, 5, 10 µmol/l 2- Isopentenyl adenine (2iP). In all experiments full strength BM with 5 µmol/l BA was used as the control medium. Previous results showed better growth with B5 vitamins than MS ones, and the meristems developed more vigorously on full strength medium with 5 µmol BA than on half strength medium with 5 µmol/l BA (RAVNIKAR & al. 1990).

Meristems (0.3–0.6 mm in length with two to four leaf primordial) grew on the growth induction medium. After 1 month they were transferred on the same medium and the callus was removed. After another month the shoots were dissected and

subcultured on elongation media: first on full strength and after four weeks on half strength mineral basal medium with complete B5 vitamins and with 1 μmol/l BA, 0.1 μmol/l NAA and 0.1 μmol/l GA_3 (KARTHA & al. 1981). In the case when the growth induction medium contained 2iP the elongation media also contained 1 μmol/l 2iP in the place of BA. After another four weeks buds were subcultured on half strength basal medium without hormones and transferred to a sterilised mixture of peat, vermiculite and soil.

After four and eight weeks of culture the diameter of the callus and caulogenic tissue was measured. The term caulogenic tissue is used for green compact mass which regenerates on the meristems after 2 weeks of culture adjacent to callus. Buds differentiate from caulogenic tissue usually after four weeks of culture. After twelve weeks the length of shoots and the de novo formation of callus was measured.

Before autoclaving all hormones were added and the pH was adjusted to 5.7–5.8. Cultures were kept at $25 \pm 2°C$. The light intensity was 70 μmol m^2s^{-1} (Sylvania Gro Lux F40T12 and fluorescent LV 20 lights) during 16 h per day.

Student's t-test were used to calculate the levels of significant differences between the control medium (from the beginning we used as the control basal medium with 5 μmol/l BA) and other media. The following symbols were used: $*0.01 < p < 0.05$, $**0.001 < p < 0.01$, $***p < 0.001$, where p means the level of significant difference between growth on control medium and other media. In the graphs I means 2× standard errors (SE). Fifteen to twenty meristems were used for each medium, and all experiments were repeated at least twice.

Results

The developed procedure of seed germination further enable us to discard seeds infected with internal pathogens before meristem isolation. The regeneration of buds on meristems was not dependent on the initial size of isolated meristems, which varied from 0.3 to 1 mm. Meristem growth was also independent of the season. On the control medium, the diameter of caulogenic tissue with buds was always 5–6 mm after 4 weeks of culture, regardless if the isolation of meristems started in autumn or in spring. On the isolated meristems, callus and caulogenic tissue appeared after 2 weeks. Callus developed on the basal part of the meristem whereas caulogenic tissue differentiated on the upper part of explant. At all BA concentrations tested, except at 0.1 μmol/l, and at two combinations of BA and GA_3 or NAA, meristems differentiated into multiple buds accompanied by callus. Callus formation was very pronounced at 10 μmol/l BA and 2iP, and specially on the medium containing BA and NAA (Table 1). The number of multiple buds was more than 5 on 10 μmol/l BA and lower at 5 and 1 μmol/l BA. At 0.1 μmol/l BA, 1–3 shoots developed, but there was no further plant development. Buds grown on the media supplemented with BA had a rosette-like growth with large individual leaves and the callus significantly redeveloped under the buds after each subculture.

Table 1

Morphogenic response of *Phaseolus vulgaris* cv. Zorin on different media after 4 weeks of culture and percentage of whole plant regeneration.

(µmol/l)	No. of buds	x̄ diameter of callus (mm)	Whole plant regeneration (%)
0.1 BA	1–3	0	0
1.0 BA	3–5	4.4±0.5	27
5.0 BA	MB	4.6±0.6	17
10.0 BA	MB	5.6±0.9	0
20 BA + 1.4 GA_3	MB	2.5±0.2	13
20 BA + 3 NAA	MB	6.6±0.9	0
1.0 2iP	1-3	3.4±0.5	37
5.0 2iP	3-5	3.6±0.7	29
10.0 2iP	0	5.5±0.6	0

MB = more than 5 buds

When the medium was supplemented with 2iP, the number of buds was reduced (Table 1). They were easy to separate and callus formation was retarded after subculture (Fig. 1). Callus formation from shoots grown on medium with 2iP was totally inhibited after 12 weeks (Fig. 2, 3a). Bud elongation was successful on the media with 1 µmol/l BA, 0.1 µmol/l NAA and 0.1 µmol/l GA_3 or 1 µmol/l 2iP, 0.1 µmol/l NAA and 0.1 µmol/l GA_3, after 8 weeks of meristem culture. After 12 weeks, the elongation of the

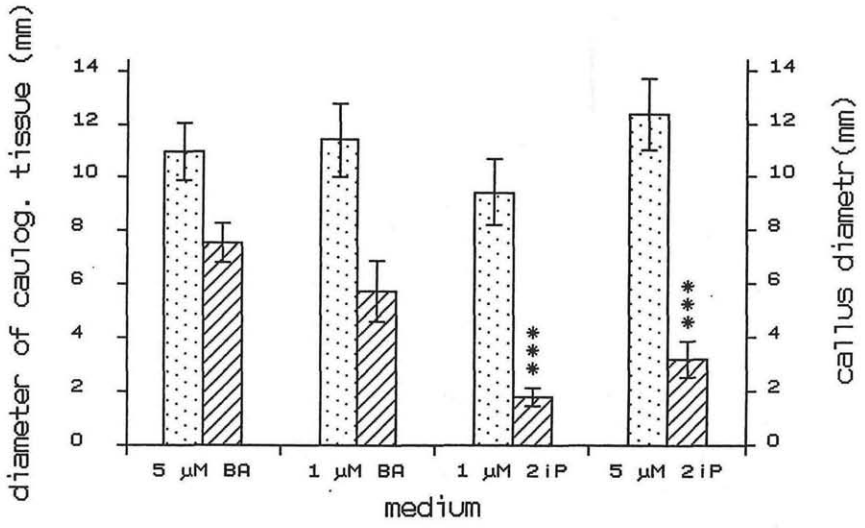

Fig. 1. The diameter of callus and caulogenic tissue developed when meristems were grown on the basal medium with different concentration of BA and 2iP, after 8 weeks of culture.

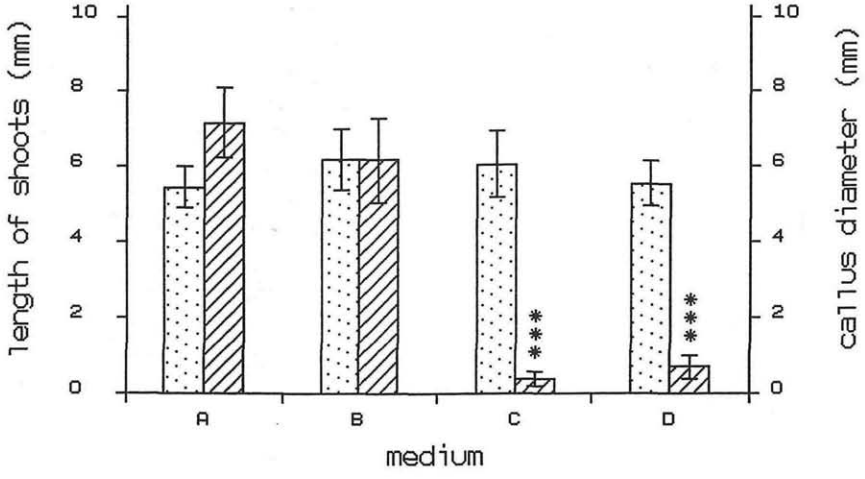

Fig. 2. Shoot length and callus diameters after 12 weeks of culture. (A) initial media were supplemented with 5 µmol/l BA and elongation media with 1 µmol/l BA. (B) initial and elongation media were supplemented with 1 µmol/l BA. (C) initial and elongation media were supplemented with 1 µmol/l 2iP. (D) initial media were supplemented with 5 µmol/l 2iP and elongation media with 1 µmol/l 2iP.

buds was better on the half strength mineral medium, with the same hormone concentration.

Shoots developed root system on the half strength medium without plant hormones after four months.

Although the buds developed on all media used, the regeneration of plantlets with developed roots able to survive the transfer to soil, was achieved only when the meristems were isolated on full strength basal medium supplemented with 1 or 5 µmol/l BA or 2iP, or with 20 µmol/l BA and 1.4 µmol/l GA_3 (Fig. 3b). The most vigorous plants developed when 1 µmol 2iP was used for initial meristem culture and was also incorporated into the elongation media. A protocol for regeneration of mature bean cv. Zorin plants suitable for routine utilisation was prepared according to these results (Fig. 4).

Discussion

We paid special attention to the influence of germination conditions and on discoloration or wrinkling of seeds during sterilization. With a 3 day darkness period we brought about that all latent bacterial diseases appeared during germination. Seeds that lost their colour or wrinkled after sterilization were discarded. SCHUSTER & al. 1987 determined different bacterial pathogens which cause brownish seeds of the dry bean, MCCLEAN & GRAFTON 1989 and FRANKLIN & al. 1991 used only unwrinkled seed for germination.

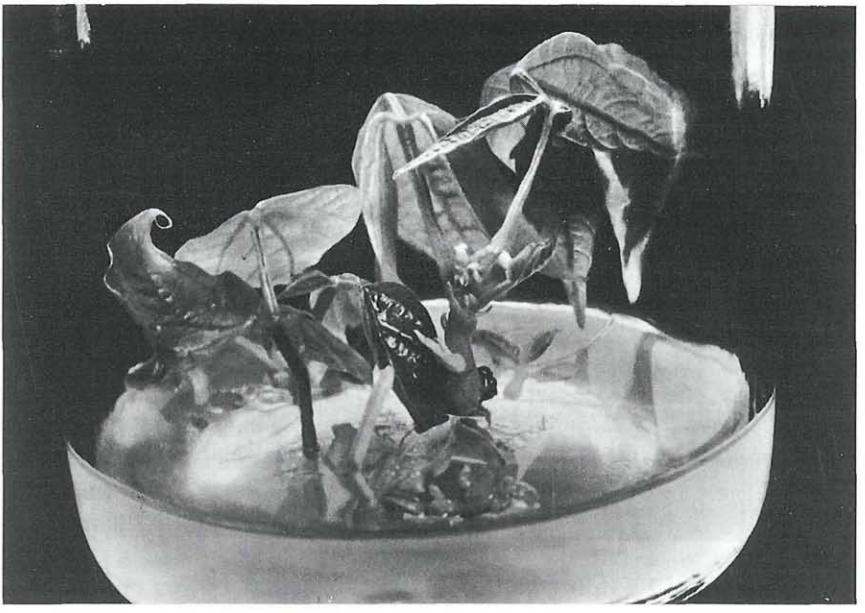

Fig. 3a. Elongated buds with roots on half strength medium without plant growth regulators, after 4 months in culture.

Fig. 3b. Bean plants regenerated from meristems three month after transfer to soil.

ILLU-STRA-TION							
Culture stage	germin-ation	initial merist. culture	bud (a) subcul-turing	bud (a) elonga-tion	shoot growth	rooting	growth to matu-rity
medium	water-agar	MSB5	MSB5	MSB5	1/2MS full B5	1/2 MSB5	soil
plant growth regula-tors (µM)	0	2iP 1	2iP 1	2iP 1, NAA 0.1, GA_3 0.1	2iP 1, NAA 0.1, GA_3 0.1	0	
sucrose (%)	0	3	3	3	3	3	
agar (%)	0.6	0.8	0.8	0.8	0.8	0.8	
culture period (days)	12	28	28	28	28	28	84

(a) callus was removed before subculturing

Fig. 4. The protocol for regeneration of mature bean cv. Zorin plants.

According to data from the literature (KARTHA & al. 1981, RUBIES-AUTONELL & al. 1982, RUBLUO & KARTHA 1985, GANTOTTI & al. 1985, SAAM & al. 1987, RUSSELL & al. 1993), BA is the cytokinin of choice in meristem culture and micropropagation of the bean. BA is used in different preferential concentration of 0.1–10 µmol/l, usually alone or in combination with IAA or NAA, rarely with IBA.

Exceptionally, KARTHA & al. 1981 reported that bean meristem differentiated into plantlets on hormone-free medium or on medium containing only the auxin NAA.

Our results show that citokinin 2iP is better than BA in initiation of buds in meristem cultures. This results is not a surprise given the diversity

of cultivars. Since the bean is recalcitrant in culture, many authors reported a cultivar dependent efficiency of bud and shoot regeneration in the different media (RUBLUO & KARTHA 1985, RUSSELL & al. 1993). Moreover RUBLUO & KARTHA 1985 showed that even at the optimal level of BA at 10 µmol/l, differential responses were observed in shoot regeneration efficiency (0–100 %).

It is common to all reports that BA in a concentration of 10 µmol/l caused multiple bud regeneration and callus formation on isolated meristems (KARTHA & al. 1981, RUBLUO & KARTHA 1985). In our experiments a higher concentration of 10 µmol/l BA had the some effect, but we could not grow complete plantlets. The problem was in the bud elongation. We obtained better results with 2iP in the elongation of buds and reduced callus regrowth in the elongation media.

Shoots developed root system on the half strength medium without plant hormones after four months, although other authors usually report that auxin are required for root initiation (KARTHA & al. 1981, RUBIES-AUTONELL & al. 1982, GANTOTTI & al. 1985, RUSSELL 1993). The percentage of plants able to survive the transfer to soil is the highest when meristems are initially cultured on media with 1 or 5 µmol/l of 2iP, up to 29 or 37% respectively. This result is quite good in comparison to the report of RUBIES-AUTONELL & al. 1982 where 17% of developed shoots completed their growth.

Conclusions

Although the buds developed on all media used, the regeneration of plantlets with developed roots able to survive the transfer to soil, was achieved only when the meristems were isolated on full strength basal medium supplemented with 1 or 5 µmol/l BA or 2iP, or with 20 µmol/l BA and 1.4 µmol/l GA_3. Our results showed that 2iP in concentrations of 1 or 5 µmol/l in the basal medium induced regeneration of the most vigorous buds. They were easy to separate and elongate on the medium with 1 µmol/l 2iP, 0.1 µmol/l NAA and 0.1 µmol/l GA_3. In the end, the highest efficiency of plants able to survive the transfer to soil, up to 37% was obtained. The rooting of shoot does not need any added hormones in the half strength basal medium. We thus worked out a system for meristem regeneration, elongation, and rooting, and prepared the protocol for production of mature bean plants according to these results.

References

BHOJWANI S. S. & RAZDAN M. K. 1983. Plant tissue culture, theory and practice. – In: Development in crop science (5). pp. 25–41. – Elsevier, Amsterdam, Oxford, New York, Tokyo.

FRANKLIN C. I., TRIEU T. N., GONZALES R. A. & DIXON R. A. 1991. Plant regeneration from seedling explants of green bean (*Phaseolus vulgaris* L.) via organogenesis. – Plant Cell Tissue Organ Cult. 24: 199–206.

GAMBORG O.L., MILLER R. A. & OJIMA K. 1968. Nutrient requirements for suspension cultures of soybean root cells. – Exp. Cell Res. 50: 151–158.

GANTOTTI B. V., KARTHA K. K. & PATIL S. S. 1985. In vitro selection of phaseolotoxin resistant plants using meristem culture of bean (*Phaseolus vulgaris* L.). – Phytopathology 75 (11): 1316–1317.

KARTHA K. K. 1982. Genepool conservation through tissue culture. RAO, A. N.: Tissue culture of economically important plants. – Proc. Int. Symp., 213–218. National University, Singapore.

KARTHA K. K., PAHL K., LEUNG N. L. & MROGINSKI L. A. 1981. Plant regeneration from meristems of grain legumes: soybean, cowpea, peanut, chickpea and bean. – Can. J. Bot. 59: 1671–1679.

MCCLEAN P. & GRAFTON K. F. 1989. Regeneration of dry bean (*Phaseolus vulgaris* L.) via organogenesis. – Plant Sci. 60: 117–122.

MOK M. C. & MOK D. W. S. 1977. Genotypic responses to auxin in tissue cultures of *Phaseolus*. – Physiol. Plant. 40: 261–264.

MURASHIGE T. & SKOOG F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. – Physiol. Plant. 15: 473–497.

QUAK F. 1977. Meristem culture and virus-free plants. – In: REINERT J. & BAJAJ Y. P. S. Plant cell, tissue, and organ culture. pp. 598–615. – Springer-Verlag, Berlin, Heidelberg, New York.

RAVNIKAR M., BENEDIČIČ D., RODE J. & GOGALA N. 1990. Regulation of organogenesis with jasmonic acid. – Acta Hort. 280: 169–172.

RICHARDSON M. J. 1979. *Phaseolus vulgaris*. – In: An annotated list of seed borne diseases, pp. 173–182. Third Edition, Dep. of Agric. and Fisheries for Scotland, Agricultural Scientific Servis, East Craigs, Edinburgh.

RUBIES-AUTONELL C., FACCIOLI G. & ZOLI C. 1982. Indagine sulla presenza di BCMV e BYMV in meristemi di fagiolo e coltura in vitro degli stessi per la produtione di plante virus-esenti. – Atti Giornate Fitopatologiche 1982 – Supplemento: 139–149.

RUBLUO A. & KARTHA K. K. 1985. In vitro culture of shoot apical meristems of various *Phaseolus* species and cultivar. – J. Plant. Physiol. 119: 425–433.

RUSSELL D. R., WALLACE K. M., BATHE J. H., MARTINELL B. J. & MCCABE D. E. 1993. Stable transformation of *Phaseolus vulgaris* via electricdischarge mediated particle acceleration. – Plant Cell Rep. 12: 165–169.

SAAM M. M., HOSFIELD G. L. & SAUNDERS J. W. 1987. In vitro propagation of dry bean from seedling shoot tips. – J. Amer. Soc. Hort. Sci. 112: 852–855.

SCHUSTER M. L., ZIEGELBEIN M. L., SMITH C. C. & SHOTKOSKI E. 1987. New types of symptoms of dry bean (*Phaseolus vulgaris*): Lightcolored seed incited by bacterial pathogens. – Fitopatol. bras. 12(4): 307–310.