Original Paper Plant Biotechnology, **20** (4), 275–281 (2003)

Somatic Embryogenesis of Cucumber (*Cucumis sativus* L) Using Seed Cuttings Obtained from Pre-Mature Fruit

Ahmed M. HASSANEIN*

Genetics Lab. Faculty of Science, South Valley University, Sohag 82524, Egypt
**Corresponding author* E-mail address: ahmedhassanein@lycos.com

Received 2 September 2002; accepted 9 June 2003

Abstract

Seed cuttings including zygotic embryo axis with its two cotyledon parts (2–5 mm^2) were extracted from green-pre-mature cucumber fruit and used as seed explants, or they were used after dividing them (after 3 days culture) into embryo axis and cotyledon parts. Their embryogenic potential increased with the increase of fruit maturity, the maximum was obtained when 3 weeks old fruits were used. The most efficient explant type was the cotyledon segments. Efficient cucumber embryogenesis was obtained using a two steps procedure: 1- somatic embryos were initiated and multiplied on Murashige and Skoog (MS) medium supplemented with 4.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 90 mM sucrose and 0.8% agar (NMM medium), 2- embryo maturation and germination were accomplished using the same medium without 2,4-D (MGM medium). The most important sign for successful transfer from the first to the second step was the appearance of slight greenish patches on the embryogenic mass (EMS), which appeared in 10 weeks.

Key words: *Cucumis sativus*, somatic embryogenesis, tissue culture, zygotic embryos.

Introduction

Cucumber is an important crop all over the word. Hybrid seeds of cucumber are preferred by growers, but multiplication of crosses, which can be simplified by somatic embryogenesis, makes the cost of their production relatively high. It means, once an initial hybrid seed is made it could be possible to clone the cross by generating somatic embryogenesis (Ladyman, 1995).

Cucumber somatic embryos have been obtained from various explant types (Handy and Chambliss, 1979; Ammirato, 1983; Chee, 1990; Lou and Kako, 1994; Ladyman, 1995). Somatic embryos obtained from immature zygotic embryos had malformed cotyledons and did not regenerate into plantlets (Kim and Janick, 1989). On the other hand, well formed embryos were obtained using cotyledonary explants, but did not include a multiplication phase of the primary embryos (Ziv and Gadasi, 1986). In other reports, both immature embryos and cotyledons of mature seeds were used as an efficient explant to obtain plantlets via somatic embryogenesis (Trulson and Shahin, 1986; Ladyman, 1995; Reichert and Zimmerman, 1996; Gawronska *et al.*, 2000; Andryskova *et al.*, 2001)

Genetically stable and regenerative embryogenic masses provided attractive tool for production of artificial seeds (Gray and Purohit, 1990) and other applications (Malepszy *et al.*, 1982; Kim and Janick, 1989; Chee, 1990, 1993; Lou and Kako, 1995, 1996). Therefore, researchers have been looking for simplifying and improving the protocols as well as establishing new ones for mass production of somatic embryogenesis with low frequency of somatic variation. Many previous reports had described protocols depend on sequential steps (Ammirato, 1983; Ladyman, 1995), e.g., initiation, multiplication, conditioning and germination. However, these are time consuming, expensive and sometimes with low frequency of somatic embryogenesis. The aim of the current research is to find a simple and reliable protocol for induction of somatic embryogenesis in cucumber, and for successful conversion of somatic embryos into plantlets for mass production. A protocol can be efficiently used for hybrid seed production, in vitro selection and transformation researches.

Materials and Methods

Explant Preparation

Cucumber fruits (*Cucumis sativus* L. cv. Balady)

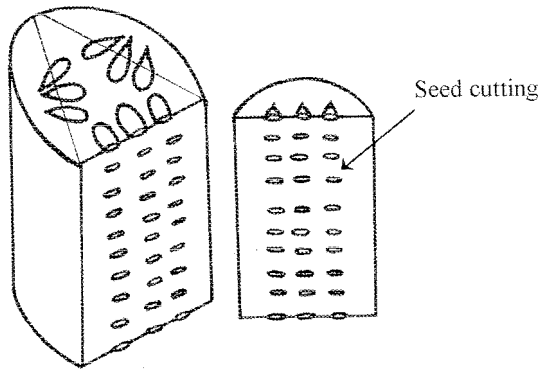

Fig. 1 Diagramatic representation of the transverse selections of a fruit to obtain seed cuttings.

about 3 weeks old were washed carefully by tap water, surface sterilized in 95% ethanol for one min and flamed for about 30 s. The two ends of the fruit were removed (2 cm each), then the remainding part of the fruit was cut into four sections, each about 2.5 cm long. Three transverse cuts were made to cut each seed inside the fruit tissue into two sections (**Fig. 1**), the three outer parts of the fruits were used to extract seed cuttings containing zygotic embryos in contact with the remainder of the cotyledon parts (2-5 mm^2). The explants within seed coat cuttings were removed from the fruit tissue and placed on NMM medium. Two developing ages of the fruits were used, when the fruits were dark green, 8-10 cm long, 2-3 cm radius, about 2 weeks old, and when seed cuttings were easily removed from the surrounded tissue of the fruits, 3 weeks old.

Embryogenic mass initiation

Seed cuttings derived from green pre-mature fruits (3 weeks old) were placed in Petri dishes, 90 mm diameter, containing 30 ml of basal medium of Murashige and Skoog (MS) salts (Murashige and Skoog, 1962), 90 mM sucrose, 8 g l^{-1} commercial agar and supplemented with 4.5 μM 2,4-D (NMM). After three days the seed coats were easily removed. Then, the cotyledon part (2-5 mm^2) of some seed cuttings were removed from the embryo axis (cotyledon explants) and some others were left as obtained from the seed coat part (seed explants). After four weeks, the explants showed embryogenic mass formation were individually transferred to glass jar (100 ml volume) containing about 30 ml NMM medium for total of 12 weeks. After ten weeks, the number of seed explants forming embryogenic mass (EMS) and the weight of EMS under the influence of fruit age were determined. The effect of embryo axis on somatic embryogenesis of cotyledon parts of the seed explants was investigated. On the NMM medium, the appearance of greenish batches on embryogenic masses was used as an indication to transfer the EMS for further development on an MS medium lacking phytohormones (MGM). To study the effect of initiation and multiplication periods on maturation and plantlet transformation, the embryogenic masses obtained from cotyledon explants were transferred to MGM medium at different periods: 6, 8, 10 and 12 weeks. Effect of different concentrations of 2,4-D (2.3, 4.5, 9 and 18 μM) and combinations between 2,4-D and other phytohormones was tested using cotyledon explants. Also, combinations between 2,4-D at either 4.5 μM or 9 μM and sucrose at either 60, 90, 120, 150 or 180 mM were tested to study their effects on embryogenesis of cotyledon explants. The sucrose was autoclaved alone and added to the medium after autoclaving.

Seed cuttings in blocks of their surrounding tissues were also placed on NMM medium to test for the effect of fruit tissue on somatic embryogenesis of seed cuttings.

General condition

Unless otherwise stated, the following conditions were applied: cultures were maintained under white florescent tubes, providing an irradiance of 40 μmol $m^{-2}s^{-1}$, in a tissue culture room at 25 ± 2 ℃ with a 16 h daily photoperiod without humidity control. The media pHs were adjusted to 5.8 before adding agar and autoclaving (20 min at 121 ℃). Three replicates were used in each experiment, and for each replicate 10 cotyledon explants were cultured. Each experiment was repeated thrice. All cotyledon parts for each tested parameter were extracted from one pre-mature fruit.

Plantlets acclimatization

Plantlets with extensive root system were washed under running tap water and potted in plastic pots in a mixture of equal amounts of soil, peat and sand. The plants were acclimatized for three weeks under tissue culture room condition. The pots were covered by plastic bags (25x40 cm) for 2 weeks. Then, two pores (each about 0.5 cm radius) were made in each bag. The pore size increased (50%) each day, and the bags were discarded by the end of the third week.

Results and Discussion

Suitability of the pre-mature fruits as a source of explants

Pre-mature cucumber fruit has smooth surface which facilitates surface disinfection. In general, large fruits or tubers are usually very easy to handle and often require flaming in ethanol prior to exci-

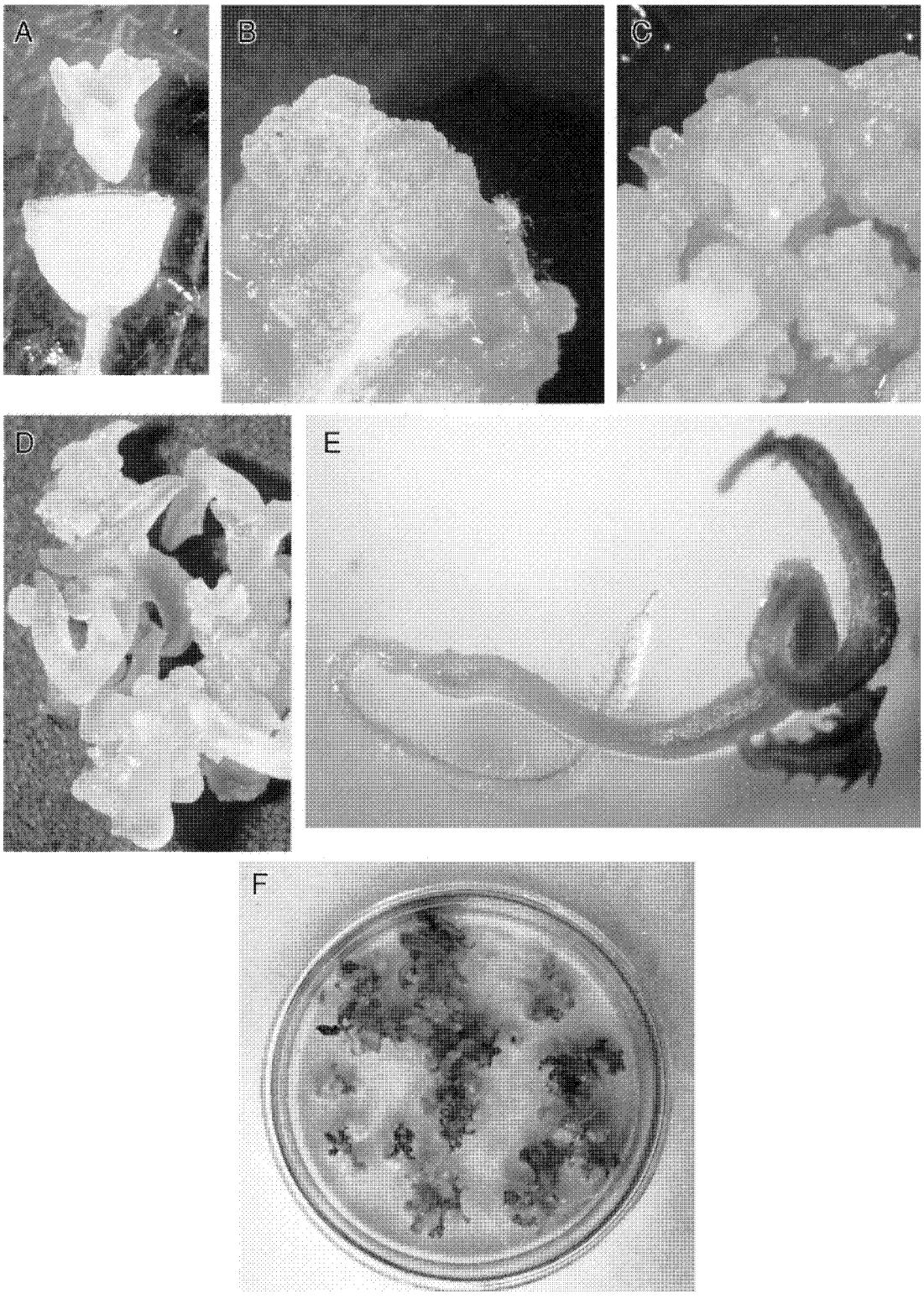

Fig. 2 Cucumber regeneration via somatic embryogenesis using seed cuttings extracted from green pre-mature fruits:

(A) Seed explants liberated from cut seed coat after three days on initiation and multiplication medium (NMM).

(B) Embryogenic mass derived from immature seed explant cultured for 4 weeks on initiation and multiplication medium (NMM).

(C) Conversion of the embryogenic mass to somatic embryos at different stages of development after 10 weeks on initiation and multiplication medium (NMM).

(D) Somatic embryos at late cotyledonary stage with others at different developmental stages on maturation and germination medium (MGM) for 2 weeks.

(E) Plantlets regeneration from somatic embryo after 6 weeks on maturation and germination medium (MGM).

(F) Regeneration of several plantlets from embryos on maturation and germination medium (MGM) for 6 weeks.

sion of internal tissue (Stafford and Warren, 1993). The seed cuttings could be obtained in high number after surface sterilization of one fruit (200-300). The direct use of seed explants (**Fig. 2A**) obtained from pre-mature fruit is more advantageous than mature old seeds. It is not only to get a great number of clean explants derived from one pollination but also to get explants never in contact with toxic disinfectants.

The data in **Table 1** show that the more suitable age of cucumber fruit was 21 days, and indicate that establishment of certain degree of seed development is important for explant extraction and somatic embryogenesis. Before and after this age, extraction of seed-explants was difficult and affected negatively the number of explants per fruit. While 200-300 seed explants were obtained from 3 weeks old fruit, 100 and 65 seed explants were obtained from 2 and 4 weeks old fruits, respectively. In three days, the explants showed enlargement inside the cut seed-coat and protrude outside, a phenomenon which made the removal of seed-coat parts very easy.

When blocks of fruit tissues including groups of seed cuttings were cultured on NMM medium, neither the seed cuttings nor the surrounded fruit tissues showed any response; e.g., they did not show neither cell division nor enlargement of the cultured tissue with time.

Table 1 Effect of fruit maturity on EMS production of seed explants cultured on NMM medium for 10 weeks.

Explant source	Percent of explants forming EMS	Weight of EMS (gm per explant)
4 weeks old fruit	29	5.8 ± 0.8
3 weeks old fruit (Control)	30	5.9 ± 0.4
2 weeks old fruit	15	5.6* ± 0.6

* Means significantly different from three weeks old fruits at $p<0.05$.

Initiation and maturation of somatic embryo

In seven days of culture on NMM medium, embryogenic mass formation appeared along the cut ends of cotyledons. The callus in four weeks was gelatinous-whitish mass and increased in size progressively with time indicating high multiplication potential. The phenotype of embryogenic mass during multiplication phase appeared in this work (**Fig. 2B**) was as described by many authors, a gelatinous matrix in which early-globular embryos were apparently suspended (Malepszy, 1988; Chee, 1990). All the explant tissues (cotyledons alone or in contact with embryo axis) changed to embryogenic mass in 8 weeks.

For 10 weeks on NMM, the embryogenic mass appeared in globular-nodular form (**Fig. 2C**) and turned greenish in some parts. The number of these forms was very high (about 1200 per cotyledon parts of one seed cutting), 25% of them followed development and established cotyledonary embryos on MGM medium. The appearance of greenish colour was taken as a sign for successful transfer of embryogenic masses to the next step of embryo maturation and germination. The greenish color was dominant after transferring the embryogenic masses to MGM medium. Green spot formation could be used to predict embryogenesis and shoot formation during in vitro culture of cucumber (Kim and Jang, 1984).

Effect of dissection of the initial explant (seed cuttings) on induction of embryo formation and germination

Since the simplicity was the first aim of this protocol, seed cuttings including zygotic embryo axises with their two cotyledon parts were used as efficient explants. After removal of embryo axis,

Table 2 Effect of seed cutting partition after 3 days with total of 10 weeks on NMM medium and 2 weeks on MGM medium on EMS production and number of embryos (at cotyledonary stage) per gm embryogenic mass.

Organ of explant	Percent of explants forming EMS	Weight of EMS per explant (gm)	No. of embryos per gm EMS
The cotyledon parts (Control)	61	7.8 ± 1.6	33 ± 2.8
Embryo axis with its two cotyledon parts	33	5.2* ± 1.2	27* ± 2.3
Embryo axis	9	1.5* ± 0.3	5* ± 1.6
Cotyledon of mature seeds	51	5.3* ± 0.3	35 ± 3.2
Hypocotyl	23	4.0 ± 0.4	10 ± 2.5

*Means significantly different from cotyledon explants at $p<0.05$.

substantial increase in the frequency of somatic embryogenesis, the weight of embryogenic mass and the number of embryos was detected on cotyledons (**Table 2**). Somatic embryos developed in low number on the embryo axis cultured independently, it may be due to decrease of embryogenic potential or due to suppression of embryogenesis by embryo axis (Lazzeri *et al.*, 1987). Difference in ability to undergo somatic embryogenesis between the different explants may be due to differences in physiological development (Griga, 1990; Lou and Kako, 1994). In the Cucurbitaceae, successful somatic embryogenesis has been obtained from numerous sources with particular emphasis on seedling cuttings, especially cotyledons and hypocotyls (Debeaujio and Branchard, 1993; Kreuger *et al.*, 1996). In this work, intact seed cuttings or their parts obtained from pre-mature fruits were used as an efficient explants for somatic embryogenesis in comparison to that obtained from hypocotyl and cotyledon explants of mature seeds which routinely used by many authors (e. g. Chee, 1990; Ladyman and Girard, 1992; Lou *et al.*, 1996).

Effect of initiation and multiplication period on somatic embryogenesis

In this work, MS basal medium with and without 2,4-D was used for embryo initiation and multiplication, as well as germination and plantlet transformation, respectively. The two steps could be accomplished in 8 weeks, but for maximum yield 16 weeks were needed. Somatic embryos were not developed on MGM medium without establishment of tubular stages on the NMM medium as was also reported by many authors (Lu and Thorpe, 1987; Blanc *et al.*, 1999). It indicates that the development of somatic embryos should reach specific stage of development on initiation and multiplication medium (NMM) before transferring them to maturate and germinate on hormone-free medium (MGM). Embryo initiation during the primary culture needed 2,4-D (NMM) and its presence during the multiplication step did not prevent their development.

Table 3 shows that the weight of embryogenic mass and the number of embryos per explant increased with increasing the incubation period on initiation and multiplication medium. After 10 weeks on NMM medium, the obtained population of somatic embryos showed a wide range of developmental stages (**Fig. 2C, D**), it may be due to the appearance of new embryogenic centers which may arise, either from cell clusters or maturing embryos (Ammirato, 1983; Von Arnold *et al.*, 2002). The most suitable period to transfer the EMS from NMM medium to MGM medium was 10 weeks, where the explants obtained relatively large embryogenic mass and they expressed great number of embryos. The germinated embryos showed growth of both shoot and root systems on MGM (**Fig. 2E, F**).

Table 3 Effect of initiation and multiplication period on EMS production, and number of embryos per cotyledon explant (at cotyledonary stage).

Number of weeks	Weight of EMS per explant(gm)	Number of embryos per explant
6	1.8* ± 0.3	10* ± 3.8
8	3.1* ± 0.4	227* ± 8.1
10 (Control)	3.5 ± 0.3	307 ± 14.4
12	3.3 ± 0.3	209 ± 11.0

*Means significantly different from cotyledon explant cultured on NMM for 10 weeks at $p<0.05$.

Effect of 2,4-D alone or in combination with other phytohormones as well as sucrose concentrations on somatic embryogenesis

Table 4 lists the results achieved for 2,4-D treatments that induced somatic embryogenesis. NMM media containing 2.3-18 μM 2,4-D were able to stimulate an embryogenic response. The maximum embryogenic frequency was achieved

Table 4 Effect of 2,4-D concentration on EMS production of cotyledon explants and on number of embryos (at cotyledonary stage) and plantlets.

2,4-D Con. (μM)	Percentage of explants forming EMS	Number of embryos perexplant	Number of plantlets per explant
2.3	41	184* ± 16.5	90* ± 9.0
4.5 (Control)	57	306 ± 22.8	148 ± 8.8
9	55	109* ± 8.5	10 ± 3.1
18	53	64* ± 13.4	6* ± 1.2

*Means significantly different from cotyledon explants cultured on NMM medium supplemented with 4.5 μM 2,4-D at $p<0.05$.

Table 5 Effect of sucrose concentration in combination with 2,4-D concentration on EMS production and number of plantlets.

Sugar con. (mM)	4.5 μM 2,4-D			9 μM 2,4-D		
	Percentage of explants forming EMS	Weight of EMS per explant (gm)	Number of plantlets per explant	Percentage of explants forming EMS	Weight of EMS per explant (gm)	Number of plantlets per explant
60	39	3.6 ± 0.6	55* ± 2.1	34	3.6 ± 0.5	7* ± 0.5
90 (Control)	58	4.0 ± 0.6	189 ± 10.6	62	3.4 ± 0.3	12* ± 2.3
120	61	3.5 ± 0.7	150* ± 7.5	58	2.5* ± 0.4	13* ± 1.0
150	56	2.9 ± 0.3	127* ± 20.0	48	2.5* ± 0.4	12* ± 0.3
180	40	1.8* ± 0.3	79* ± 20.8	46	2.5* ± 0.3	8* ± 0.6

*Means significantly different from cotyledon explants cultured on NMM medium and supplemented with 4.5 μM 2,4-D and 90 mM sucrose at $p<0.05$.

with explants cultured on NMM supplemented with 4.5 μM 2,4-D, where somatic embryogenesis was induced at a 57% frequency.

The number of plantlets obtained on MGM medium was affected by the 2,4-D concentration in NMM medium, 4.5 μM 2,4-D was the best for obtaining the maximum number of cucumber plantlets. The increase in the 2,4-D concentration decreased strongly the number of plantlets (**Table 4**). It is in accordance with another study of Ladyman (1995), he reported that somatic embryo multiplied in the presence of 5 μM 2,4-D resulted in normal plants, where the 2,4-D concentration higher than 8 μM gave rise to morphologically abnormal seedlings. Lou *et al.* (1996) reported that the increase in 2,4-D concentration linearly enhanced the frequency of embryo formation and embryo yield up to peak at 8 μM, thereafter, the yield was negatively affected.

It is well known that, exogenous auxins for initiation of somatic embryo formation are essential prerequisite (Carman and Campbell, 1990), but improvement cucumber regeneration may depend on other hormones or non-hormonal factors (Ladyman and Girard, 1992; Lou and Kako, 1994). In this work, combination between 2,4-D and other phytohormones significantly reduced the number of somatic embryo obtained from the cotyledon explant of pre-mature fruits (Data not shown). Also, comparison between the effect of higher and lower concentrations of sucrose (**Table 5**) indicated that, the relatively higher concentrations (120-180 mM) were more important for induction and germination of somatic embryos than the lower one (60 mM).

Transfer the plantlets to the soil

A period of 3 weeks for humidity acclimatization under plastic pages was essential prerequisite for successful transfer of the plantlets from Petri dishes to the soil, where 85% of plantlets were successfully transferred. During the acclimatization period, the plantlets underwent physiological and morphological adaptations enabling them to develop typical terrestrial plant water control. The phenotype of transferred plants was normal as plants obtained from seed germination under the same condition.

Conclusion

Comparison between this work and others (see references) indicates that the most factors affecting somatic embryogenesis of cucumber are cultivars (e.g. Lou and Kako, 1994) and the source of plant material. In addition, other factors such as initiation and multiplication period of somatic embryos, 2,4-D and sucrose concentrations affect the efficiency of somatic embryogenesis in cucumber.

References

Ammirato, P. V., 1983. Embryogenesis. In: Evans, D. A. *et al.* (Eds.): Handbook of Plant Cell Culture. Vol. 1, pp. 82-123. Mac Millan, New York.

Andryskova, L., Klems, M., Havel, L., Vlasinova, H., 2001. Initiation and maintenance of cucumber (*Cucumis sativus* L.) embryogenic cell suspension. 17 International Con. Plant Growth. Subs. Brno, Czech Rep.

Blanc, G., Michaux-Ferriere, N., Teisson, C., Lardet, L., Carron, M. P., 1999. Effects of carbohydrates addition on the induction of somatic embryogenesis in *Hevea brasiliensis*. Plant Cell, Tissue Organ Cult., **59**: 103-112.

Carman, J. G., Campbel, W. F., 1990. Wheat-factor affecting somatic embryogenesis in wheat. In: Bajaj, Y. P. S. (Eds.): Biotechnology in Agriculture and Forestry. Plant Protoplast and Genetic Engineering. Vol. 13, pp. 68-87. Springer- Verlag, Heidelberg,.

Chee, P. P., 1990. High frequency of somatic embryogenesis and recovery of fertile cucumber plants. HortSci., **25**: 792-793.

Chee, P. P., 1993. Transformation in cucumber (*Cucumis sativus* L.). In: Bajaj, Y. P. S. (Eds.): Biotechnology in

Agriculture and Forestry. Plant Protoplast and Genetic Engineering. Vol. 23, pp. 215-227. Springer- Verlag, Heidelberg.

Debeaujon, I., Branchard, M., 1993. Somatic embryogenesis in Cucurbitaceae. Plant Cell, Tissue Organ Cult., **43**: 91-100.

Gawronska, H., Burza, E., Malepszy, S., 2000. Zygotic and somatic embryos of cucumber (*Cucumis sativus* L.) substantially differ in their levels of abscisic acid. Plant Sci., **157**: 129-137.

Gray, D. J., Purohit, A., 1990. Somatic embryogenesis and development of synthetic seed technology. Crit. Rev. Plant Sci., **10**: 33-61.

Griga, M., 1990. Some factors affecting somatic embryogenesis efficiency in soybean (*Glycine max* L. Merr.). Biol. Plant., **35**: 179-189.

Handly, L. W., Chambliss, O. L., 1979. In vitro propagation of *Cucumis sativus* L. HortSci., **14**: 22-23.

Kim, S. G., Jang, J. R., 1984. Regeneration of plants from callus tissue of cucumber (*Cucumis sativus* L.) seedling cotyledons. Plant Physiol., **75**: 1-1.

Kim, Y. H., Janick, J., 1989. Somatic embryogenesis and organogenesis in cucumber. HortSci., **24**: 702-702.

Kreuger, M., Meer-W-Van-der, Postma, E., Abbestee, R., Raaijmakers, N., Horst, G. J., 1996. Genetically stable cell lines of cucumber for the large-scale production of diploid somatic embryos. Physiol. Plant., **97**: 303-310.

Ladyman, J. A. R., Gerard, B., 1992. Non-hormonal factors that improve the multiplication and development of somatic embryos of cucumber (*Cucumis sativus* L.). Acta. Hort., **300**: 233-236.

Ladyman, J. A. R., 1995. Somatic embryogenesis in cucumber (*Cucumis sativus* L.). In: Bajaj, Y. P. S. (Eds.): Biotechnology in Agriculture and Forestry. Somatic Embryogenesis and Synthetic Seed. Vol. 31, pp. 151-163. Springer-Verlag, Heildelberg.

Lazzeri, P. A., Hildebrand, D. F., Sunega, J., Williams, E. G., Collins, G. B., 1987. Soybean somatic embryogenesis: effects of nutritional, physiological and chemical factors. Plant Cell, Tissue Organ Cult., **10**: 209-220.

Lu, C. Y., Thorpe, T. A., 1987. Somatic immature embryo of *Picea glauca*. J. Plant Physiol., **128**: 797-302.

Lou, H., Kako, S., 1994. Somatic embryogenesis and plant regeneration in cucumber. HortSci., **29**: 906-909.

Lou, H., Kako, S., 1995. Role of high sugar concentration in inducing somatic embryogenesis from cucumber cotyledons. Sci. Hort., **64**: 11-20.

Lou, H., Kako, S., 1996. Influence of sucrose concentration on in vitro morphogenesis in cultured cucumber cotyledon explants. J. Hortic. Sci., **71**: 499-502.

Lou, H., Obara-Okey, P., Tamaki M., Kako, S., 1996. Influence of sucrose concentration on in vitro morphogenesis in cultured cotyledon explants. J. Hortic. Sci., **71**: 497-502.

Malepszy, S., 1988. Cucumber (*Cucumis sativus* L.). In: Bajaj, Y. P. S. (Eds.): Biotechnology in Agriculture and Forestry. Vol. 6, pp. 277-292. Springer Verlag Berlin, Heidelberg, New York.

Malepszy, S., Niemirowicz, K., Wiszniewska, J., 1982. Cucumber (*Cucmis sativus* L.) somatic embryogenesis in vitro. Acta Biologica, **10**: 218-220.

Murashige, T., Skoog, F. A., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., **15**: 472-497.

Reichert, N. A., Zimmerman, N. J., 1996. Somatic embryogenesis versus organogenesis in cucumber: developing a laboratory exercise. HortTechnol., **6**: 84-86.

Stafford, A., Warren, G., 1993. Plant Cell and Tissue Culture. pp. 82-100. John Wile & Sons, Chicester, New Yourk, Brisbane, Totonto, Singapore.

Trulson, A. J., Shahin, E. A., 1986. In vitro plant regeneration in genus *cucumis*. Plant Sci., **47**: 35-44.

Von Arnold, S., Sabala, I., Bozhkov, P., Dyachok, J., Filonova, L., 2002. Developmental pathway of somatic embryogenesis. Plant Cell, Tissue Organ Cult., **69**: 233-249.

Ziv, M., Gadasi, G., 1986. Enhanced embryogenesis and plant regeneration from cucumber (*Cucumis sativus* L.) callus by activated charcoal in solid/liquid double-layer culture. Plant Sci., **47**: 115-122.