Factors Affecting Embryoid Formation in Hypocotyl Callus of *Solanum melongena* L.

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Factors influencing embryogenesis were investigated on the hypocotyl calluses of a cultivar (Shinko-Yaenari) of *Solanum melongena* L. When actively growing calluses were conditioned by NAA, occurred embryogenesis which was observed by the appearance of green spots (GS). 1. A wide range of the concentration of NAA (4~20 mg/l) in the medium was effective for GS formation, while 7~9 mg/l was the optimal. 2. Duration of the callus culture with high NAA medium gave influences to the yield of embryoids; longer duration increased the percentage of the GS forming calluses, while shorter duration enhanced the GS number per callus. 3. Calluses on the 2 mg/l NAA medium kept the callus growth for more than 19 passages (14 months). When the callus was conditioned by the high NAA medium, embryoids were formed. Calluses transferred to NAA-free medium induced organogenesis. The organogenetic ability decreased at the early passages and was lost finally but the embryogenetic ability was kept for a longer period. Plants embryogenetically regenerated from early passages were diploids except one plant, but those from later passages contained many polyploids. 4. The embryogenesis was also influenced by the condition of explants; long explant pieces taken from non-elongated hypocotyls yielded many GS.

KEY WORDS : *Solanum melongena*, tissue culture, regeneration, embryogenesis, organogenesis, hypocotyl, callus.

Introduction

One of the important contributions of tissue culture technique to agriculture is the regeneration of plants of the same genotype. In the regeneration from callus tissues, two morphogenetic types are known; organogenesis and embryogenesis. The embryogenesis may be much more useful for large scale propagation, and knowledges about the factors influencing the embryoid yield may contribute for agricultural investment. As for *Solanum melognena*, Yamada et al. (1967) and Kamat and Rao (1978) have obtained successful plant regeneration from callus tissues by giving appropriately balanced IAA and Kinetin in media. Matsuoka and Hinata (1979) pointed out the hypocotyl callus of several Japanese cultivars induced either organogenesis or embryogenesis depending upon the NAA concentrations given in a medium. Fassuliotis et al. (1981) and Gleddie et al. (1982) succeeded in the plant regeneration from callus and protoplasts taken from leaves.

The present experiment was carried out to refine the effect of NAA on the yield of embryoids by giving various duration of the treatment. The effect of explant tissues was also investigated.

Materials and methods

A cultivar 'Shinko-Yaenari' was used as the material, which differentiated either adventitious shoots or embryoids depending upon the concentrations of NAA in the

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medium; low or zero level of NAA induces organogenesis, 1~2 mg/l NAA keeps callus growth and about 8 mg/l NAA induces embryoids (Matsuoka and Hinata 1979).

The basic medium comprised Murashige and Skoog's (1962) mineral salts and organic components (Matsuoka and Hinata 1979). Hypocotyl explants taken from aseptically germinated seeds were cultured for callus induction on the agar-solidified basic medium containing NAA (α -naphthalene acetic acid); the NAA medium. After callus formation, the callus was transferred to the basic medium without NAA for better differentiation of embryoids. At the transfer, the callus mass taken into a liquid basic medium was stirred in a glass tube to make dispersed cell colonies. Then, the cell colonies were mixed with agar basic medium in a liquid state (ca. 50°C) and plated onto a thin layered basic medium that solidified beforehand at the bottom of a 100 ml flask. The other routine techniques were detailed in the previous report (Matsuoka and Hinata 1979).

Formation of green spots (GS) was observed to indicate embryoid differentiation. Two parameters were presented; the percentage of calluses with GS to total calluses observed (GS-callus percent) and the average number of GS per the GS forming calluses (GS number).

Results

1. NAA concentration

Concentrations of NAA in the NAA medium were varied from 4 to 12 mg/l in 1 mg intervals. Hypocotyl pieces were cultured on the medium in dark at 25°C for 9 or 12 weeks, and then the calluses were transferred to the NAA free medium. Each plot was replicated with 10 explants.

GS differentiation is presented by the GS number at 3, 6 and 9 weeks after the transfer (Fig. 1). GS differentiated in every plot so far given. The favorable concentration was inferred to be 7~9 mg/l.

Fig. 1. Effect of NAA concentration on the GS formation. Explants were cultured on the high NAA medium for 9 (○) or 12 (●) weeks and transferred to the NAA free medium. GS number was observed at 3 (a), 6 (b) and 9 (c) weeks after the transfer.

Fig. 2. Effect of the duration of the high NAA culture on the GS-callus percent (a) and GS number (b). Calluses cultured for varied weeks (7-14 weeks; vertical) on the high NAA medium were transferred to the NAA free medium and the GS formation was observed weekly (horizontal).

The GS number increased by 3 weeks after the transfer but decreased thereafter. As for the duration on the NAA medium, the 12 week culture induced greater number of GS at the early observations, whereas the 9 week culture kept the GS forming ability for a longer period and it induced greater number at the later observations.

2. Culture durations on NAA medium

Hypocotyl explants were cultured on the NAA medium (10 mg/l) for varied durations from 7 to 14 weeks. Then the callus was transferred to the NAA free medium and the GS induction was observed weekly for 12 weeks. Each plot was replicated by 40 explants.

The GS-callus percent and the GS number are presented on Fig. 2a and Fig. 2b, respectively. The trend of the GS induction differed between short duration of the NAA culture (the short NAA culture; shorter than 10 weeks) and long duration (the long NAA culture; longer than 10 weeks). In the short NAA culture, the first appearance of GS was earlier, but the GS-callus percent was less than 50%. In contrast, in the long NAA culture the calluses for more than 11 weeks formed GS sporadically per se, and when they were transferred to the NAA free medium the GS-callus percent increased rapidly up to 80%, though it decreased thereafter. The GS number altered in different way. In the short NAA culture, it increased more than 15 per callus, whereas in the long NAA culture it was less than 10. In every duration the time at which the maximum number of GS was obtained was 14~20 weeks after the inoculation of the explant. After 20 weeks, the GS number decreased. Some of the GS turned to calluses again by themselves and some others were embedded in the surrounding callus cells.

3. Regeneration ability in long term culture

Hypocotyl explants were cultured on NAA (2 mg/l) medium, on which callus continued the growth without embryogenesis nor organogenesis. The calluses were subcultured successively for 19 passages (14 months). At the transfer, which was made every 3 or 4 weeks, a callus mass was divided into a few pieces, and one of them, being about 20 mg in fresh weight, was placed to the fresh NAA medium of the same composition for the successive culture. The remaining callus pieces were placed on a high NAA medium (8 mg/l NAA) and on a low NAA medium (0.02 mg/l NAA + 2 mg/l 6-BA), when the callus stocks were checked on the ability of embryogenesis and organogenesis, respectively. Thirty callus stocks originated from different seeds were cultured respectively. To check the embryogenetic ability, 20 stocks were sampled at random from the 30 stocks, and the organogenetic ability was checked for 10 stocks which were taken at random from the 20 stocks.

These checks were made at every passage except 4, 13, 14, 16 and 18 passages. The calluses placed on the high NAA medium were again transferred to NAA free medium after 6 weeks to secure good embryoid formation.

Changes of regenerating ability along the successive culture are presented on their GS-callus percent (Fig. 3a) and the GS number (Fig. 3b). As for embryogenesis, the GS-callus percent was near to 100 percent in the early passages but it decreased later on. The GS number was not so high in the initial passages but it increased rapidly in a few passages, and it decreased gradually thereafter. Calluses taken from the early passages formed GS rapidly, while those of the later passages showed retarded formation of GS.

Fig. 3. Changes of the regenerating ability of embryogenesis (●) and organogenesis (▲) for the calluses in long term culture. (a): the GS-callus percent and (b): the GS number.

plants. Variation of ploidy was observed among the plants that originated from the same callus mass. Diploid plants were all normal in the shape of leaves, flowers and fruits, but tri- and tetraploid plants showed some kinds of irregularities in morphology. In a mixoploid (18-3), the observed cells involved diploid cells (ca. 50%), triploids (15%), tetraploids (10%) and aneuploids. Its morphology was irregular and its distorted fruits contained very few seeds.

4. Conditions of explants

In the preliminary test conducted before the above mentioned experiments, we have recognized that the yield of GS are greatly affected by the condition of the explant.

Table 1. Chromosome numbers of the plants derived, *via* GS, from callus at different passages in successive cultures

Number of passages	Callus-plant number	Number of chromosomes (2n)
Plant from seed	—	24
1	1-1	24
3	1-1	mixoploid
3	2-1	24
7	13-1	24
7	13-7	24
8	1-7	36
8	14-8	24
8	14-15	24
8	14-17	48
11	18-3	mixoploid
11	18-8	36
11	18-17	36

The organogenetic ability altered differently. It decreased rapidly within a few passages. The number of adventitious shoot was very low and finally became nil.

Some of the GS due to embryoids at 1, 3, 7, 8 and 11 passages were nursed to obtain whole plants. Of 130 embryoids in total taken to the nurse culture, 12 whole plants were obtained. Root tips taken from fully expanded plants were pretreated with 8-hydroxyquinoline and stained with acetocarmine for the observation of chromosomes. As shown in Table 1, the plants regenerated from the early passages were diploids except one mixoploid, but those from the 8 and 11 passages had only 2 diploids out of 7

Table 2. Effect of the condition of explants: Middle or large pieces (the small one was omitted) were taken as the explants from upper, middle or lower portion of hypocotyls which were short or elongated

Hypocotyl length	Portion	Size of explant	GS-callus percent	GS number
Short	Upper	Middle	30	2.0
		Large	90	8.9
	Middle	Middle	20	3.5
		Large	70	6.3
	Lower	Middle	30	4.7
		Large	90	6.4
Elongated	Upper	Middle	20	1.0
		Large	90	2.8
	Middle	Middle	20	3.0
		Large	70	2.3
	Lower	Middle	50	2.6
		Large	40	5.5

Two kinds of hypocotyls grown in dark were used; short (3 cm long at 5 days after germination) or elongated (6 cm at 10 days). Small (0.2 mg in fresh weight), middle (0.8 mg) or large (2.1 mg) pieces were taken from upper, middle or lower portion of each hypocotyl. Ten pieces of explants for each plot in the combination of the conditions were cultured on the NAA (10 mg/l) medium for 11 weeks. The calluses were transferred onto the NAA free medium and the GS formation was observed.

Calluses made from the short hypocotyls yielded many GS, as compared with the elongated ones. The size of explants also influenced the GS yield. The small explants hardly grow into callus and data for the middle and the large explants are presented in Table 2. The large explants were favourable for GS formation. The position of hypocotyl from which explants were taken, however, gave little influences on the GS formation.

Explant pieces of the comparable ages, sizes and positions were macerated with chromic acid (Sasahara 1971) and the number of cells in a piece was estimated. Figure 4 shows the relation between the average cell number per explant and the average GS number per callus. GS were obtained when an explant piece was composed of more than 2×10^3 cells in the present condition of culture. The elongated hypocotyl contained large and vacuolated cells more frequently than the short did. GS were effectively differentiated from the small cells rich with cytoplasm.

Fig. 4. Relation between the GS number and the average cell number in an explant piece which was taken from short (○) or elongated (●) hypocotyl.

Discussion

In the previous paper we have pointed out that the callus originated from the hypocotyl

of a cultivar, 'Shinko-Yaenari' differentiates either adventitious shoots or embryoids depending upon the concentration of NAA in the medium; low or zero level of NAA induces organogenesis, 1~2 mg/l NAA keeps callus growth and about 8 mg/l NAA induces embryogenesis (Matsuoka and Hinata 1979). Gleddie *et al.* (1982) reported that 10 mg/l NAA was good for embryogenesis in their leaf tissue culture experiments. Although 7~9 mg/l NAA was optimum for the present material, the range of effective concentration was relatively wide.

Conditions of the explants also influenced the ability of embryogenesis. Juvenile cells produced a good number of embryoids. It can be concluded that the embryogenesis is caused when an actively growing callus is conditioned by a high NAA medium.

The calluses under successive culture kept the embryogenetic ability. They lost it gradually in very long passages. This seems, at least partly, due to the genetical changes of cells since the polyploid production increased in the plants that regenerated from calluses of the later passages. Such tendency was observed in many materials (Murashige and Nakano 1966, Bayliss 1980). In contrast to the embryogenetic ability, the organogenetic ability was lost at rather earlier passages. Organogenesis seems to be more influenced by several factors such as cell interactions, cell activities etc.

Thorpe (1980) has pointed out that there are at least three requirements for *de novo* organized development; (1) cell dedifferentiation, (2) cell interaction and (3) reaction of specific signals. NAA in the present experiment exercised two kinds of effects to the callus; one was to induce callus growth and the other was to induce embryoids, which might be comparable to the cell dedifferentiation and the reaction of specific signals, respectively. The signals of NAA, however, has not yet been fully analyzed. The two effects of NAA may be, sometimes, opposite each other: When the callus growth is enhanced by NAA, the formed GS is expected to dedifferentiate again by the NAA, but when the effective NAA is not enough, the GS formation will not be completed. The effect of the culture duration in the present experiment could be explained in this view point. When the long culture duration completed the conditioning for most of the callus increasing the GS-callus percent, the high concentration of NAA in the callus dedifferentiated the GS again and decreased the GS number. On the other hand, the short duration could not completed the conditioning for many calluses, resulting in the low GS-callus percent, while the conditioned callus could differentiate many embryoids. An appropriate conditioning to cause a balanced development between the callus growth and the GS formation should be looked for to achieve high GS yield.

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Literature Cited

- BAYLISS, M. W. 1980. Chromosomal variation in plant tissues in culture. *Internat. Review Cytol. Suppl.* 11 A : 113~144.
- FASSULIOTIS, G., B. V. NELSON and D. P. BHATT 1981. Organogenesis in tissue culture of *Solanum*

- melongena* cv. Florida Market. Plant Sci. Letters 22: 119~125.
- GLEDDIE, S., W. KELLER, G. SETTERFIELD 1982. *In vitro* morphogenesis from tissues, cells and protoplasts of *Solanum melongena* L. in Plant Tissue Culture 1982, Maruzen, Tokyo, p.139~140.
- KAMAT, M. G. and P. S. RAO 1978. Vegetative multiplication of eggplant (*Solanum melongena*) using tissue culture techniques. Plant Sci. Letters 13: 57~65.
- MATSUOKA, H. and K. HINATA 1979. NAA-induced organogenesis and embryogenesis in hypocotyl callus of *Solanum melongena* L. J. Exp. Bot. 30: 363~370.
- MURASHIGE, T. and R. NAKANO 1966. Tissue culture as a potential tool in obtaining polyploid plants. J. Hered. 57: 115~118.
- MURASHIGE, T. and F. SKOOG 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15: 473~497.
- SASAHARA, T. 1971. Genetic variations in cell and tissue forms in relation to plant growth. II. Total cell surface area in the palisade parenchyma and total cell surface area: Total nitrogen content in relation to photosynthetic activity in *Brassica*. Japan. J. Breed. 21: 61~68.
- THORPE, T. A. 1980. Organogenesis *in vitro*: Structural, physiological, and biochemical aspects. Intern. Rev. Cytol. Suppl. 11 A: 71~111.
- YAMADA, T., H. NAKAGAWA and Y. SINOTO 1967. Studies on the differentiation in cultured cells. I. Embryogenesis in three strains of *Solanum* callus. Bot. Mag., Tokyo 80: 68~74.

ナス (*Solanum melongena* L.) 胚軸カルスからの胚状体形成に
関与する条件について

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Solanum melongena L. の一栽培品種「新交八重成」を用いて、胚軸由来カルスからの胚状体形成条件について検討した。カルスを高濃度 NAA で一定期間培養すると緑点 (GS) を形成するので、これを指標とした。

1. NAA は 4~20 mg/l の濃度範囲で GS を形成したが、7~9 mg/l が最適であった。2. 高濃度 NAA 培地に置く期間によって、GS 形成量は異なり、長期間培養すると GS を形成するカルスの割合は高くなるが GS の数は多くない。一方短期間培養では GS を形成するカルスの割合は低いが GS を形成したカルス当りの GS 数は増加した。3. NAA 2 mg/l の培地上では、カルスが増殖を続けるので、19 回 (14 カ月) 植え継いで、その分化能の変化を調べた。継代したカルスを高濃度 NAA に移植すると胚状体の GS を形成し、低濃度 NAA に移植すると不定芽を形成した。不定芽形成能は継代初期に急速に低下したが、胚状体形成能は比較的高く維持された。胚状体の GS から植物体を育てたところ、継代初期のカルスからの再生体には 2 倍体が多く、後期のものからの再生体には倍数体が多かった。4. GS 形成数は外植体切片の大きさにも依存し、未伸長の短い胚軸から大きな切片をとると、得られる GS 数が多かった。