Evaluation of competent embryo production and conversion potential in callus cultures initiated *in vitro* from leaf explants of six sweet potato [*Ipomoea batatas* (L.) Lam] genotypes

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Summary

The aim of this study was to evaluate *in vitro* embryogenic potential of selected sweet potato [*Ipomoea batatas* L. (Lam.)] cultivars. Leaf blade and petiole from *Ipomoea batatas* plants (genotypes Rojo Blanco, White Jewel, White Star, Nemanete, Papota, and IIA-TIB 10) grown in pots and *in vitro* cultures were used for experiments as primary explants. The potential of embryogenic callus induction and somatic embryo development was evaluated for the cultures grown on the media supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP), and/or abscisic acid (ABA), under different light conditions (photoperiod, constant darkness).

The cultivars appeared to differ with regards to embryogenic response of explants, somatic embryogenesis progression as well as embryo development and plant regeneration potential. The highest frequency of somatic embryogenesis was found for cv. Rojo Blanco and embryo development resulted in fully regenerated, healthy plants. In other cultivars, somatic embryo induction occurred, but embryogenesis has been arrested at the globular stage. White Star appeared to be the only completely recalcitrant cultivar which did not show any embryogenic response.

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1. Introduction

Ipomoea batatas L. (Lam.), the common name – sweet potato or camote, belongs to the morning-glory family (*Convolvulaceae*). It originates from Central and South America and the Pacific Islands but where specifically the wild species came from has not been determined yet. It is a perennial, monoecious plant with storage roots growing up to 3 kg in weight. Funnel-shaped, light violet or white blossom occurs on climbing vines. Although native to the American Tropics, sweet potato has been introduced and cultivated in many tropical and subtropical countries where, due to its nutritional value, it became an important food crop, especially in India, China, Philippine Islands, and the South Seas Islands (1). For optimal growth and yield, *Ipomoea batatas* requires tropical or temperate climatic conditions. Although in tropical areas, sweet potatoes flower and cross-pollinate easily, seeds are rarely used for propagation purposes since plants do not come true from seed. In Northern areas where plants never flower, it is propagated by vegetative means, from transplants produced by bedding mother roots, or from rooted cuttings. Sweet potato is subjected to injury from a large number of diseases that may attack the young plants in the hot bed or the growing crop in the field or may cause a decay in storage. The worst of these are stem rot, black rot, foot rot, soft rot or ring rot, and root rot (2).

The storage roots of sweet potato constitute the source of carbohydrates, proteins, ascorbic acid, vitamin A, riboflavin, β-carotene, calcium, iron, and minerals (3). Sweet potato is regarded as a valuable raw material for producing alcohol (methanol, ethanol). It is used as an alternative source of energy and substrate for fuel, tactic acid, acetone, butyl alcohol, vinegar, and yeast production (4).

Ipomoea batatas is a hexaploid (2n = 90) and its putative ancestors, according to Magoon et al. (5) were tetraploid and diploid species. Magoon et al. (5), showed that contemporary sweet potato genome consists of three genomes of closely related parental species. Also Butler et al. (6), who characterized microsatellite sequences in selected diploid and polyploid *Ipomoea* species, indicated that *Ipomoea batatas* most likely originated from hybridization of two wild cultivars – *Ipomoea trifida* and *Ipomoea tiliacea* – followed by polyploidization.

Male sterility, sex incompatibility and hexaploidy of sweet potato result in a limited possibility of improvement of existing cultivars utilizing classical breeding methods (4,7,8). For the past decade, efforts have been made to genetically improve and enrich sweet potato cultivars using modern *in vitro* techniques such as somatic hybridization, cytoplasmic recombination, and DNA transfer technology (4,7-12). As reported from the Indonesia-Indochina and Middle and South American Centers of Diversity, sweet potato, or cultivars thereof, is reported to tolerate bacteria, dis-

ease, drought, fungus, hydrogen fluoride, high pH, lateritic soil, low pH, mycobacteria, nematodes, peat, savanna, virus, and weeds (13). Both existing sweet potato germplasm as well as other *Ipomoea* species which can be donors of unique traits (genes) in future may play a critical role in the improvement of sweet potato. For example, some desirable features were identified in *I. trifida* and *I. littoralis* resistant to sweet potato weevil (*Cylas* spp.), scab [*Elsinoe batatas* (Saw.)], and black root (*Ceratocystis fimbriata*) (14).

Plant tissue cultures and regeneration techniques are useful for the production of somaclonal variants and transgenic plants (15). One of the most valuable methods of plant regeneration *in vitro* is somatic embryogenesis in which totipotency, the natural regeneration potential characteristic of plants, is fully exploited/utilized. This method is successfully used to propagate plants of many species: *Theobroma cacao* (16,17), *Digitalis obscura* (18), *Daucus carota* (19), *Camellia sinensis* (L.) (20), *Pennisetum glaucum, Sorghum bicolor* (L.), Moench (21), *Coffea arabica, Cichorum, Abies alba, Solanum tuberosum, Lycopersicon esculentum, Vitis vinifera* (22), *Hordeum vulgare* (23). The technology of highly repetitive, efficient plant propagation *via* somatic embryogenesis has become the method of choice for producing transgenics of numerous plant species (cultivars) (24,25) as well as artificial seeds (26,27). Somatic embryos are used in cryopreservation, the easiest and most economical method to preserve and storage a genetic material of many plant species, including sweet potato (28).

Somatic embryogenesis *in vitro* is one of the methods used for sweet potato propagation. It can be induced directly from many types of explants, e.g. shoot apical buds, shoot apices, apical shoot meristems, leaves, petioles, roots, steams or callus tissue (10,25). Despite of that, however, there has not been established any optimized protocol of plant regeneration *via* somatic embryogenesis that could be used for efficient genetic transformation of various cultivars of *Ipomoea batatas*. This paper reports on the protocol for efficient plant regeneration of *Ipomoea batatas* cv. Rojo Blanco that is currently used in our laboratory for transformation research.

2. Material and methods

2.1. Plant material

Six cultivars of sweet potato, *Ipomoea batatas* (L.) Lam., were used in this study. They were obtained from two sources: Rojo Blanco was kindly provided by Dr. C. S. Prakash, Center for Plant Biotechnology Research, Tuskegee University, AL, while Nemanete, White Jewel, White Star, Papota, IIA-TIB 10 – by Dr. R. L. Jarret, USDA, Southern Regional Plant Introduction Center, Griffin, GA and USDA, Beltsville Agricultural Research Center (BARC), Beltsville, MD.

The primary explants used in the experiments originated from the plants grown in pots or *in vitro* shoot cultures maintained on multiplication medium (MM) consisting of MS (Murashige and Skoog) inorganic salts and vitamins (29), *myo*-inositol 100 mg/L, GA_3 5 mg/L, sucrose 30 mg/L, and Phytagel 3.5 mg/L (all chemicals from Sigma-Aldrich). Initially, the induction of embryogenic callus cultures, somatic embryo development as well as plant regeneration were carried out according to the modified protocols from the laboratory of Dr. C. S. Prakash (30). The leaf blade discs and petiole cuttings were used as explants (tab.). During the first stage of the experiment, the explants were grown in constant darkness, at 26°C, on one of the three variants of callus production medium (CPI): MM medium supplemented with KCl (2.237 g/L) and combination of 2,4-D (1.0, 2.21, or 2.46 mg/L) and 0.25 mg/L BAP. The growth regulator concentrations have been chosen basing on preliminary experiments performed according to the protocol provided by Dr. Prakash (data not shown). After two weeks, the explants from Rojo Blanco cultivar (in the case of other cultivars stage II was omitted) were transferred onto CPII medium supplemented with 2,4-D (0.44 mg/L) and grown for another week in dark. Subsequently, they were transplanted onto somatic embryo inducing medium (EP) containing ABA at the concentration of 2.5 mg/L and cultured for one week in dark and then another week under 16 h photoperiod.

Table

The number of primary explants of different cultivars of *Ipomoea batatas* used for somatic embryogenesis induction

Medium	Nemanete	Rojo Blanco	Papota	White Star	White Jewel	IIA-TIB10
CPI E (1.00 mg/L 2,4-D + 0.25 mg/L BAP)	142	189	63	38	47	66
CPI G (2.21 mg/L 2,4-D + 0.25 mg/L BAP)	142	182	62	54	48	59
CPI N (2.46 mg/L 2,4-D + 0.25 mg/L BAP)	144	174	62	56	47	52

Next, the callus tissue was subcultured onto EP medium without ABA and after 2-week culture in dark it was transferred to 16 h photoperiod. The cultures were maintained on EP medium in a 2-week transfer cycle. The plantlets that developed from embryogenic callus cultures were rooted on MM medium and then transplanted in pots of soil.

2.2. Histological analysis

Microtome sections of callus tissue were prepared according to Kononowicz et al. (31,32). The tissue samples were fixed for 24 h in FAA (formalin : glacial acetic acid : 50% ethanol, 1:1:8, v/v/v), rinsed for 5 min in 30% ethanol, and then for another 5 min – in distilled water.

After dehydration in ethanol series (15, 50, 70, 96, and 99,8%), the preparations were transferred to ethyl alcohol + xylene mixtures, then to pure xylene and, finally, to Paraplast *Plus* (Sigma).

Six μm thick microtome sections were stained with Safranin O and Fast Green or Hematein and Orange G according to Jensen (33).

2.3. Cytogenetic analysis of root meristems of regenerated plants

The root tip meristem treated with 0.05% solution of colchicine for 4 h were fixed in Carnoy (ethyl alcohol: glacial acetic acid, 3:1 v/v) for 24 h at 4°C. Dry ice squashed preparations were made after the Feulgen staining (1 h hydrolysis in 4 N HCl, at room temperature, incubation in Schiff's reagent for 40 min) (31,32).

3. Results and discussion

It is well documented that both exo- and endogenous auxins are involved in regulation of cell growth and proliferation, differentiation of vascular tissue, apical domination as well as root formation. It was shown by several authors (7,16-19,21) that the use of 2,4-D or NAA at the early stages of somatic embryogenesis induction in carrot, cacao, millet and sorghum was critical for successful regeneration of plants. Similarly, in the case of sweet potato (15,18,34), a high level of 2,4-D (10 μM) and 3% sucrose are required for induction and maintenance of embryogenic potential. However, at the next stage of regeneration, the amount of auxin in the medium must be reduced or it has to be removed from the medium, while cytokinins should be added in order to promote growth and development of somatic embryos (19,18). According to Padmanabhan et al. (18), addition of exogenous auxin, such as 2,4-D, at the early stage of culture stimulates accumulation of endogenous IAA. This contributes to cell proliferation and maintenance of their state of embryogenic competence. Withdrawal of auxins at the next stage of culture leads to a decrease of endogenous IAA level, which results in the establishment of inner polarization of developing embryos. On the other hand, ABA is used to promote somatic embryo formation and to increase embryogenesis frequency (24). In this study, to induce callus formation and growth three variants of medium (varying in 2,4-D concentration) were evaluated (tab.). It was shown that the me-

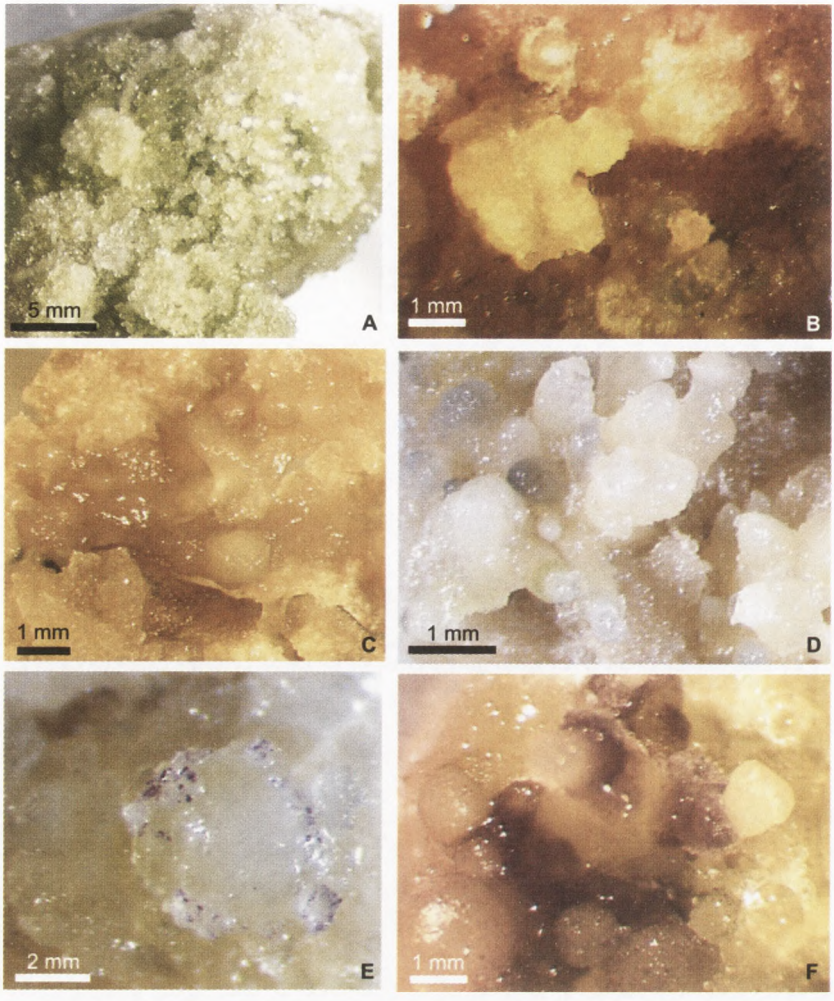

Fig. 1. Early stages of somatic embryogenesis in callus culture of sweet potato. A and B) non-embryogenic callus; C) embryogenic callus; D and F) the globular stages of somatic embryos; E) anthocyanins accumulation in embryogenic callus cells.

dium with the highest 2,4-D concentration (variant N) was optimal for callusogenesis promotion for all studied cultivars.

At the early stage of culture, similarly to Zheng et al. (24), the formation of two different types of callus, embryogenic (E) and non-embryogenic (NE), was observed both at leaf **blade and leaf petiole explants. The embryogenic callus was cream color, dense, with nodular/granular structure, and growing slowly, while non-embryogenic callus** – white-grayish color, loose, with friable structure, and growing very quickly. In the surface cell layer of embryogenic callus accumulation of anthocyanins was observed (**fig. 1E**). Biosynthesis of anthocyanins is characteristic

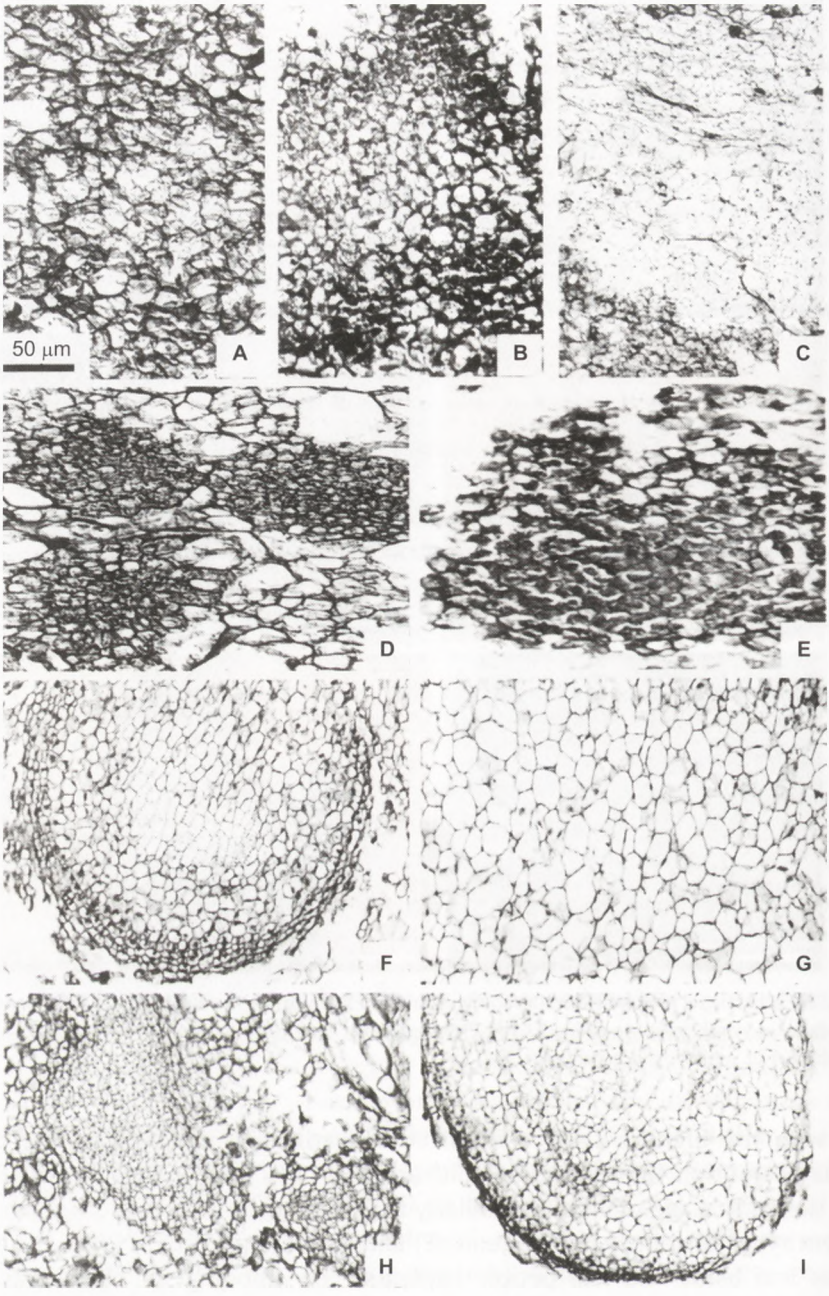

Fig. 2. Histology of *Ipomoea batatas* embryogenic callus. A) type I cells; B) type II cells; C and G) type III cells; D, E, and H) type IV cells; meristemoid localized at the center portion of callus tissue sample; F and I) type IV cells; meristematic centers localized at the periphery of callus sample.

for cells of tissues that differentiate in darkness. Similar phenomenon was observed in *Vitis vinifera* and cell suspension cultures of sweet potato cv. Ayamurasaki (34,35). Non-embryogenic callus appeared at the early stage of culture and its growth was very fast. At the beginning, it appeared mainly at the edges of explants as while, spherical structures, but later it spread over the explant surface. Based on histological analysis of E callus samples of *Ipomoea batatas* cv. Rojo Blanco and White Jewel, four types of cells were identified (fig. 2): type I: cells with relatively thick cell walls localized at the edges/periphery of callus tissue; type II: large cells with thinner then type I walls; type III: the largest cells, highly vacuolated, with relatively thin walls, localized in the inner part of the callus clumps; type IV: small, initial (meristematic) cells, with dense cytoplasm and relatively large nuclei. They form meristematic centers localized at the edges/peripheries of callus or meristemoids localized deep insight of callus clumps. This type of cells was previously observed by Quiroz-Figueroa et al. (22) in *Coffea arabica*. In the central part of the callus tissue, vascular element differentiation was also observed (fig. 2 C and F). Based on computer vision analysis of somatic embryos of *Ipomoea batatas*, Padmanabhan et al. (27,36) described five basic types of somatic embryos at torpedo and cotyledonary stages: "perfect", "near-perfect", "with limited meristematic activity", "with disrupted internal anatomy", and "proliferating". Only the first two types were competent to develop and form normal plantlets. The others were found incompetent. The types "perfect" and "near-perfect" were characterized by meristematic cells with very dense cytoplasm which form dome at the apical part of shoot and with very well developed vascular tissue. The other three types of embryos, due to abnormalities in apical meristem structure, which appeared at the early torpedo stage, were unable to continue further growth and development (27,36). Similarly, Nickle and Yeung (37,38) pointed out that lack of conversion into mature somatic embryo in carrot was caused by the abnormalities in shoot apical meristem development resulting from its disruption. The time of first somatic embryos appearance varied for different cultivars – the shortest, ca one month, was observed for cultivar White Jewel, two-three months for cv. Papota and IIA-TIB 10, and up to four months for cv. Rojo Blanco. Macroscopic analysis showed that at the initial stages of development, somatic embryos had the shape of small, smooth spheres and formed small clusters. Later, at the torpedo stage, they became slightly elongated and finally reached cotyledonary stage. The plantlets that were obtained through somatic embryogenesis were transferred to pots and to greenhouse conditions, and they became fully-grown, phenotypically normal plants. All the above-mentioned stages of regeneration and development were observed only in cv. Rojo Blanco cultures (fig. 1 and 3).

Seven out of eleven regenerated plants came from explants grown in dark, while four – under 16 h photoperiod (fig. 3 F). All plants originated from the leaf blade explants were grown on CPI N medium at the stage I of culture.

It is known that direct shoot regeneration does not influence genetic stability at cytogenetic level. However, if callus tissue is used for regeneration, an increase in

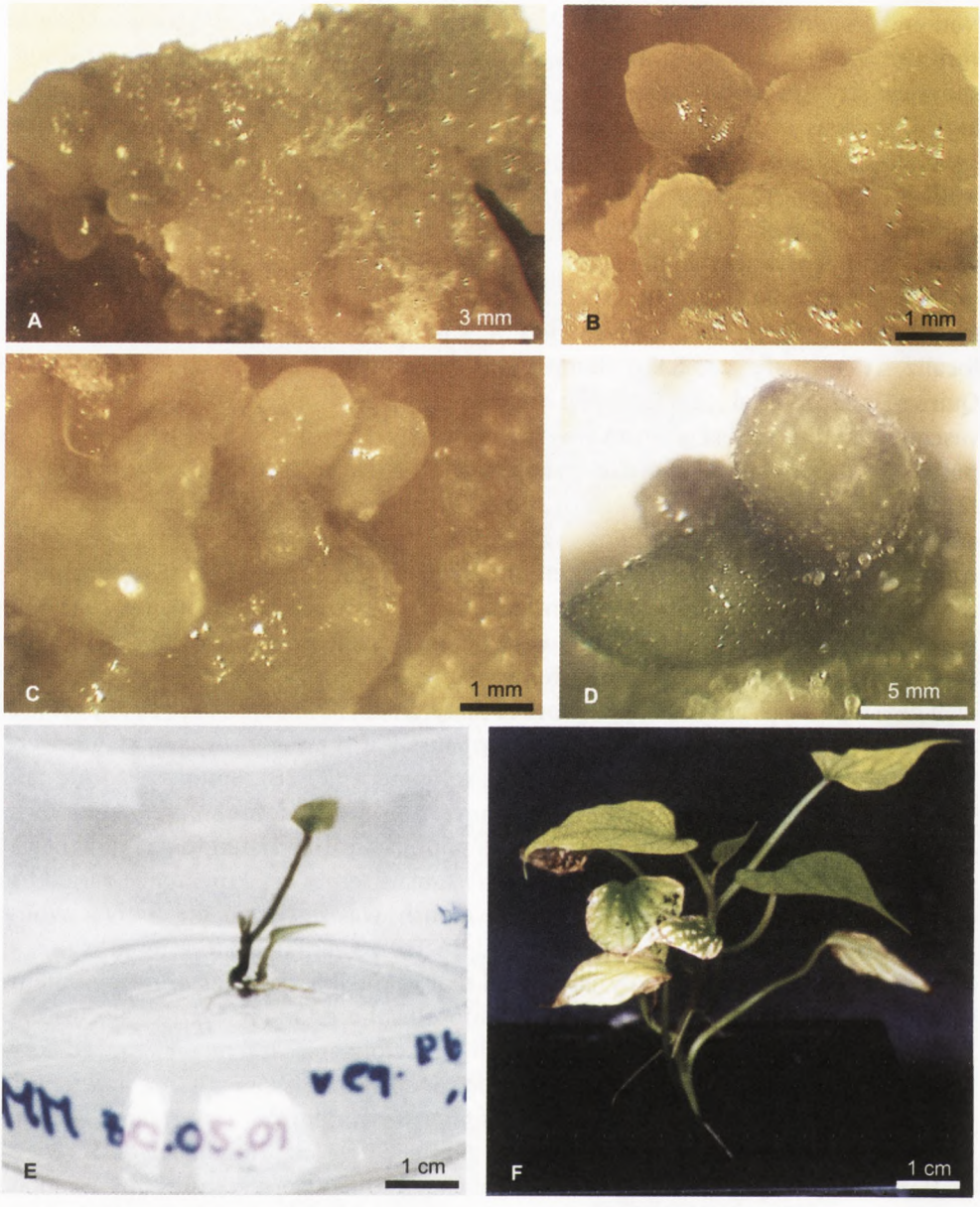

Fig. 3. Successive stages of somatic embryogenesis and plant regeneration. A – D) the torpedo stages of somatic embryos; E) sweet potato plantlet on MM medium; F) *I. batatas* plant regenerated *via* somatic embryogenesis *in vitro* from leaf explant.

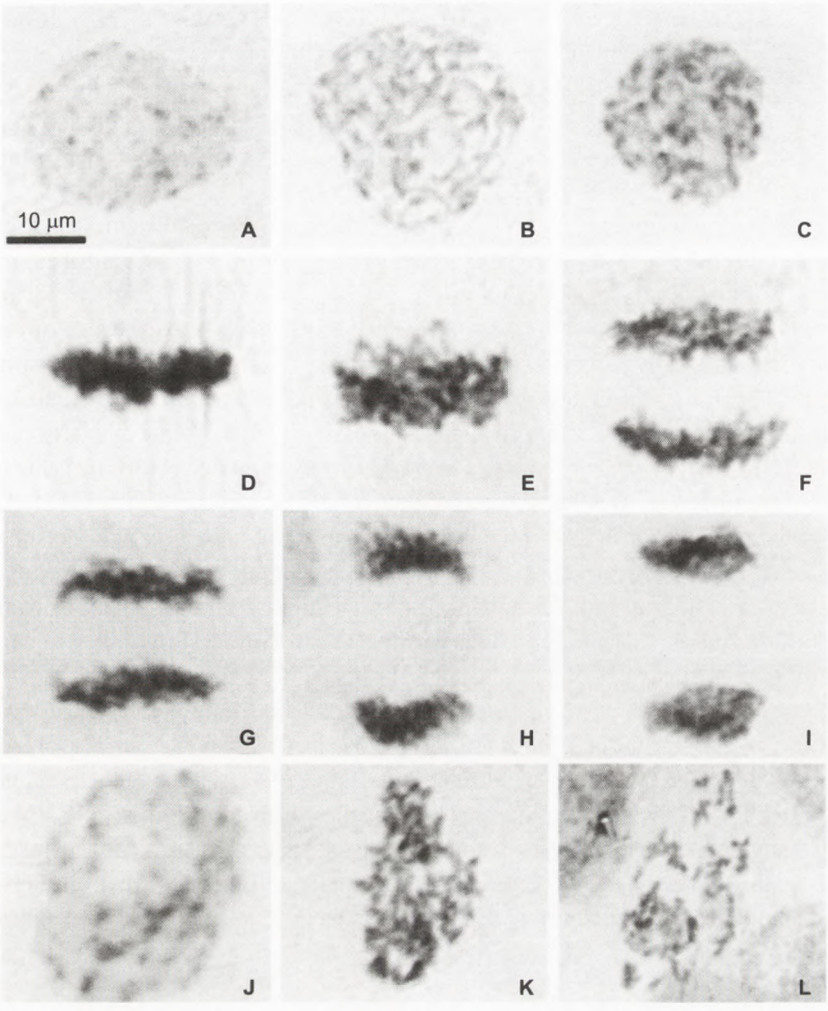

Fig. 4. Mitotic divisions in root tip meristematic cells of *I. batatas* plants regenerated *via* somatic embryogenesis *in vitro*. A and J) interphase nuclei; B and C) prophases; D) metaphase; E-G) consecutive stages of anaphase; H and I) telophases; K and L) metaphase plates.

chromosomal instability is possible (39-41). In this study no aberrations in the chromosome number (polyploidy/aneuploidy) were observed in the regenerated plants (fig. 4).

In the case of cultivars Papota, White Jewel, and IIA-TIB10 somatic embryo development was arrested at the globular stage and no further progress in embryo development was observed. White Star and Nemanete genotypes appeared to be insensitive to the applied hormonal treatment, furthermore no morphogenic response was observed and White Star was the only cultivar to produce only non-embryogenic cal-

lus. Similar results for cv. White Star were obtained by Zheng et al. (24). It has to be emphasized, however, that Padmanabhan et al. (18) (using apical shoot meristems as primary explants) and Bressan et al. (42) reported plant regeneration *via* somatic embryogenesis for this cultivar. In the case of Nemanete, embryogenic callus which appeared at the onset of culture, was later eliminated (overgrown) by non-embryogenic callus.

The results obtained indicate a gradual decrease in morphogenic potential of callus lines together with the age of culture, in the subsequent subcultures. Initially, this decrease was observed as a limited ability of embryo transformation into plantlets (Rojo Blanco), but after several-month culture, the production of embryos decreased dramatically (Papota, IIA-TIB 10, White Jewel). This phenomenon is explained by physiological changes that occur in long-term plant tissue cultures and the changes accompanying them in gene expression (18). In old embryogenic callus lines which produce incompetent somatic embryos, desensitization of tissue, resulting from accumulation of auxins and their metabolites may take place (18). Another reason for inhibition of further development of somatic embryos may be the abnormalities in development of shoot apical meristems which may appear already at torpedo stage (36). Nickle and Yeung (37,38) and Padmanabhan et al. (36) found out that this phenomenon was responsible for the loss of conversion ability in carrot somatic embryo cultures.

Macroscopic analysis showed that the cultivars Rojo Blanco and White Jewel produced the greatest number of somatic embryos. In the case of sweet potato, as of many other plants, the nature of donor-plants (age, physiological status) is very important both for initiation, course, and completion of organogenesis (15,25) and for somatic embryogenesis. The differences in the regeneration potential of leaf explants resulting from leaf age may be due to the differences in the levels of endogenous auxins, cytokinins and ABA. Earlier experiments by Al-Mazrooei et al. (25) and Porobo Dessai et al. (15) showed that best results were obtained from leaf blades from 1-4 leaves from the top of the shoot. In the present research the explants also came from the youngest (1-5) leaves of donor-plants. It is known that there is a close correlation between plant genotype and its ability to regenerate *in vitro*, including somatic embryogenesis (15). The results presented here are in agreement with this general rule.

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