REGENERATION OF *PHARBITIS NIL* FROM IMMATURE EMBRYOS BY SOMATIC EMBRYOGENESIS

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ABSTRACT

Immature embryos of *Pharbitis nil* Chois. were used for the study. They were isolated from previously sterilized fruits and afterwards transferred to Murashige and Skoog (MS) growth medium with 0.8% agar. Immature embryos were cut across their axis. After 6-8 weeks of cultivation in the injury place (hypocotyl-root region) somatic embryos were formed. These embryos were isolated and each of them was transferred into a separate tube containing MS supplemented with naphthaleneacetic acid (NAA; in concentration $0.1 \text{ mg} \cdot \text{dm}^{-3}$) and gibberellic acid (GA_3 ; in concentration $0.5 \text{ mg} \cdot \text{dm}^{-3}$). Under these conditions about 26% of somatic embryos regenerated into complete plants. Two-three weeks after the photoperiodic induction flowers appeared on these plants. A few weeks after pollination normal seeds were developed from these flowers.

KEY WORDS: Embryo formation, flowering, micropropagation, morning glory, tissue culture.

INTRODUCTION

Short day *Pharbitis nil* Chois. cv. Violet belongs to *Convolvulaceae* family. Since in *Pharbitis nil* flower buds formation is initiated by a single induction cycle (16 hour in the dark), it is very convenient model system to investigate the mechanism of flowering (Vince-Prue & Gressel 1985). The influence of plant hormones (Nakayama & Hashimoto 1973, Amagasa & Suge 1987, Galoch et al. 1995), light (Shinozaki 1972), temperature (Takimoto et al. 1960) and other factors (e.g. nitrogen and carbohydrate nutrition) (Ishiooka et al. 1991) on flowering of *P. nil* had been examined. The above mentioned investigations were conducted under *in vivo* or *in vitro* conditions, on shoot apex cultures. So far it has been well documented that the photoperiodic induction of flowering is phytochrome-dependent (Vince-Prue 1994). To determine precisely function of this photoreceptor in the regulation of flowering of *P. nil*, phytochrome mutants or phytochrome transgenic plants would be useful. Unfortunately, so far there has been no success in obtaining the mutants of *P. nil* in which one of the phytochrome pools (labile; phyA or stable; phyB) is blocked (Vince-Prue 1994). Also the first attempts of *P. nil* transformation using *Agrobacterium tumefaciens* were unsuccessful (Araki et al. 1989). The only result was transgenic callus resistant to kanamycin. This failure was caused by the lack of an efficient regenerative system (Araki et al. 1989). The earlier attempts of *P. nil* regeneration from

immature embryos and from cotyledon protoplasts were unsuccessful (Matsubara & Nakahira 1966, Messerschmidt 1974). The appearance of embryogenic callus on fragments of cotyledons, leaves and hypocotyl of *P. nil* was observed by Li and O'Neill (1993). However, it was not accompanied by the maturation of embryos (Li & O'Neill 1993). There are only very few reports concerning completely regenerated *P. nil* plants from immature embryos by somatic embryogenesis (Jia & Chua 1992; Li & O'Neill 1993).

MATERIALS AND METHODS

The investigations were conducted on immature embryos of *Pharbitis nil* Chois. Seeds of *P. nil* were treated for 50 minutes with the concentrated sulphuric acid, washed for a few hours in running tap water and then, after the whole night soaking they were sown into pots containing a mixture of perlite and sand (1:1). The germination and cultivation of plants were conducted on the continuous white light, at 26°C . After 5 days the obtained seedlings were treated with a single inductive photoperiod (8 hours of light/16 hours of darkness). Then, they were grown for 5-6 weeks under non-inductive conditions (on continuous light), after which the appearance of fertile flowers was observed. The flowers were pollinated mechanically using a brush with soft hair, and after the successive 4 weeks immature fruits were picked up. The obtained fruits contained 4 seeds on the average.

The fruits were surface-sterilized with 70% EtOH for 30 seconds and then with 10% NaOCl for 20 minutes and afterwards they were washed 4 times with sterile distilled water.

Abbreviations:

NAA – α -naphthaleneacetic acid, GA_3 – gibberellic acid, MS – Murashige & Skoog (1962)

Fig. 1. Somatic embryogenesis from immature embryos of *Pharbitis nil*. A 4-week old embryo of *Pharbitis nil* just after isolate (an arrow shows place of injury) (A). Formation of somatic embryos on zygotic embryo without (B) and with cotyledons (C). A single isolated somatic embryo (D). Bars: 2 (A), 2 (B), 3 (C) and 10 mm (D), respectively.

Fig. 2. Stages of plant regeneration from somatic embryos of *Pharbitis nil*. (A) Somatic embryo with roots and shoot (A). Completely regenerated plant with fully developed leaves (B). Regenerated plant with flower bud (C). Flowered plant regenerated from somatic embryo (D). Bars; 10 (A), 10 (B), 5 (B) and 15 mm (D), respectively.

TABLE 1. Frequency of somatic embryogenesis.

	total	number of embryos indicate somatic embryogenesis	max. number of somatic embryos on one zygotic embryo
embryos with cotyledons	65	36	28
embryos without cotyledons	24	18	9
cotyledons only	24	0	0

The embryos were isolated from the seeds and after beeing cut along their axis they were transferred onto the Petri dish containing 10 ml of Murashige and Skoog growth medium each (3% sucrose, 0.8% agar, pH = 5.7) (Murashige & Skoog 1962). The explants were transferred every 4 weeks onto fresh medium – until somatic embryos were obtained. These embryos were transferred one by one to test tubes containing MS medium supplemented with α -naphthaleneacetic acid (NAA; in concentration of 0.1 mg·dm⁻³) and gibberellic acid (GA₃; in concentration of 0.5 mg·dm⁻³) and were cultivated under variable lighting condition (16 h of light/8 h of darkness) at 26 °C. After 6 weeks fully regenerated plants were transferred to pots containing the mixture of perlite and sand (1:1 ratio), the plants were exposed to inductive photoperiod and were cultivated until mature seeds were formed.

RESULTS AND DISCUSSION

Eight weeks after the isolation of immature embryos in the injury place (Fig. 1A) somatic embryos appeared on 55% of the embryos (Fig. 1B). On each zygotic embryo 1 – 28 somatic embryos appeared. Removing the cotyledons from zygotic embryos increased frequency of somatic embryogenesis even up to 75% (Fig 1C; Tab. 1). In the case of many plant species, including *P. nil* (Li & O'Neill 1993) somatic embryogenesis is induced by auxin, especially by 2,4-D. This study prooves that somatic embryos of *P. nil* may be formed without the presence of auxins, but only after zygotic embryo injury. However, this process was additionally stimulated by synthetic auxin, naphthaleneacetic acid (NAA). Also Jia and Chua (1992) established the inductive influence of NAA on the formation of somatic embryos of *P. nil*.

As we observed in our study the frequency of zygotic embryos exhibiting the somatic embryogenesis could be compared to that one obtained by Jia and Chua (1992). However, in our experiments we applied 30-times lower concentration of NAA (0.1 as compared with 3 mg·dm⁻³) and twice lower concentration of sucrose (3 as compared to 6%) in the medium. Moreover, considering their size (7-10 mm), which according to Jia & Chua (1992) corresponded to the 3rd category, the embryos obtained by us exhibited a greater ability to embryogenesis.

The isolated somatic embryos were transferred one by one to test tubes (Fig. 1D) where 56% of them developed roots, however, among them only 12% developed both roots and shoots (Fig. 2A & 2B). On many regenerating plants we could observe necrosis of shoot apex, anomaly developed, dwarf leaves or tapping of stems. In order to counteract the dying of shoots the amount of Ca²⁺ in the medium was doubled, which increased the percentage of normally regenerating plants over twice.

In order to determine the ability of regenerated plants to flower, they were exposed to photoperiodic induction (8 h of

light and 16 h of darkness). 2-3 weeks after the induction flower buds appeared on the investigated plants (Fig. 2C) and normally developed flowers were formed. The plants transferred to non-sterile conditions survived almost in 100%, flowered (Fig. 2D) and developed seeds able for germination.

In the present work an efficient method of *P. nil* regeneration from immature embryos has been described. We are going to use this method for regeneration of *P. nil* transformed with phytochrome genes.

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REGENERACJA *PHARBITIS NIL* Z NIEDOJRZAŁYCH ZARODKÓW
NA DRODZE SOMATYCZNEJ EMBRIOGENEZY

STRESZCZENIE

Do badań stosowano niedojrzałe zarodki *Pharbitis nil* Chois. Po ich izolacji z uprzednio sterylizowanych owoców przenoszono je na pożywkę wzrostową Murashige i Skoog'a (MS) zestaloną 0.8% agarem. Niedojrzałe zarodki nacinano wzdłuż ich osi. Po 6-8 tygodniach hodowli w miejscu zranienia (w rejonie pomiędzy hypokotylem a korzonkiem zarodkowym) dochodziło do formowania się zarodków somatycznych. Zarodki te izolowano, a następnie każdy z nich przenoszony był do osobnych probówek zawierających pożywkę MS uzupełnioną kwasem naftalenooctowym (NAA, w stężeniu $0.1 \text{ mg} \cdot \text{dm}^{-3}$) i kwasem gibberelowym (GA_3 , w stężeniu $0.5 \text{ mg} \cdot \text{dm}^{-3}$). W tych warunkach około 26% somatycznych zarodków regenerowało w kompletne rośliny. Około 2-3 tygodnie po poddaniu ich fotoperiodycznej indukcji obserwowano pojawianie się kwiatów. Kilka tygodni później, po zapyleniu, dochodziło do powstawania normalnych nasion.

SŁOWA KLUCZOWE: Tworzenie zarodków, kwitnienie, mikropropagacja, *Pharbitis nil*, kultury tkankowe.