A new approach to direct somatic embryogenesis in *Medicago*

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Received December 18, 1990/Revised version received April 4, 1991 – Communicated by N. Amrhein

Abstract. A highly efficient system for direct somatic embryogenesis is described. Leaf sections originating from young trifoliate leaves of *Medicago falcata* line 47/1-5 and *Medicago sativa* line No2/9R, directly produced embryos after cultivation in liquid BSIV induction medium. In comparison with indirect somatic embryogenesis the system omits the callus stage and thus allows shortening of the process of somatic embryogenesis in alfalfa by 35-40 days. It permits the avoidance of secondary changes occurring during the process of dedifferentiation. A modified BS/3M medium containing Polyethylene Glycol 6000 promoted embryo development from globular up to torpedo stage. It was clearly shown that 2.5% Polyethylene Glycol stimulated this process for both *M. falcata* 47/1-5 and *M. sativa* No 2/9R. Maturation of torpedo stage embryos was carried out on solidified or liquid abscisic acid-containing medium. A 30 μM abscisic acid concentration was optimal in allowing one embryo to yield one plant. Somatic embryo conversion to plants and plant regeneration was performed on Murashige and Skoog medium. Regenerated plants showed a normal morphology.

Abbreviations: ABA = Abscisic acid; BS = Medium of Gamborg et al. (1968); COT = Cotyledone stage embryos; 2,4-D = 2,4-dichlorophenoxyacetic acid; FW = Fresh weight; GA3 = Gibberellin A3; MS = Medium of Murashige and Skoog (1962); PEG = Polyethylene Glycol; POLY = Polyembryos.

Introduction

High-frequency somatic embryo production has been obtained for several alfalfa genotypes either on solidified media or in suspension culture (McCoy and Bingham 1977; Lupotto 1986; Stuart and Redenbaugh 1987). However, for all embryogenic systems developed so far an indirect origin of somatic embryo formation was reported excluding protoplast systems (Kao and Michayluk 1980; Dijak and Brown 1987).

Our previous results (Denchev 1987) had shown that the highly embryogenic lines from *M. falcata* and *M. sativa* in comparison with the numerous genotypes selected so far (Atanassov and Brown 1986; Brown and Atanassov 1985; Seitz and Bingham 1986) produced somatic embryos on solid induction medium in parallel to callus formation. A stringent correlation had been established between the stage of development of the initial explant, the concentration of 2,4-D and the process of dedifferentiation and differentiation in vitro (Denchev and Atanassov 1988). The present study was undertaken with the following aims:

i) to establish a new system for direct somatic embryogenesis in *Medicago* clones possessing a high embryogenic capacity;

ii) to determine the factors that influence the induction of globular somatic embryos with respect to their development to torpedo stage as well as their further maturation and conversion to vigorous plants.

Materials and Methods

Plant material. *Medicago falcata* line 47/1-5 and *Medicago sativa* line No 2/9R selected for their high regenerative potential were used in the experiments. Plants from both lines were propagated by nodal cuttings on a 0.7% agar MS medium at 26°C under a 16/8 h photoperiod. The clones obtained in this way were used for the screening of direct embryogenic response. Plant preparation. Young trifoliate leaves (5-6 mg FW) were separated from 30-day-old cuttings. They were cut into small pieces in liquid BS5 medium supplemented with 0.5 g/l casein hydrolysate, 0.5 g/l myo-inositol and 0.3% sucrose (BS5).

Induction of direct somatic embryos. After washing with fresh medium the material was inoculated into 100 ml flasks containing a defined volume of BS5 liquid induction medium which is a BS5 modified with 18 μM 2,4-D, 0.93 μM kinetin, 4.4 μM adenine and 32.5 μM glutathione. For somatic embryo induction 15 mg of the leaf material were added to 15 ml of medium. The cultures were maintained on rotary shaker (100 rpm) under 16/8 h photoperiod at 1 800 lux, 22°C and 75% humidity from 10 to 30 days. The effect of the induction was detected after transferring the entire number of directly induced globular embryos into BS5/SM developmental medium (BS5 supplemented with 0.3% maltose instead of sucrose) containing 1% PEG for 40 days.

Somatic embryo development. For embryo development, explants containing globular embryos and embryos which had already separated from the explants were collected by centrifugation (2000 rpm for 5 min) washed 2-3 times with fresh BS5 medium and transferred into BS5/SM medium supplemented with 1, 2.5, 5 and 10% PEG 6000 (Serva) for 20 days.

Somatic embryo maturation and plant regeneration. For embryo maturation randomly-picked torpedo stage embryos were placed on MS solid medium containing different concentrations of ABA (10, 20, 30, 40 and 50 μM). For each treatment 20 embryos were plated in 9 cm Petri dishes and treated with ABA for 2 weeks. In separate experiments randomly-picked torpedo stage embryos were inoculated in liquid BS5 medium supplemented with 30 μM ABA for one, two, three or four weeks. For final evaluation, the somatic embryos were transferred to MS.
medium containing 27 μM GA₃ (MGG). All media used were sterilized by autoclaving at 120°C for 20 min except for GA₃ which was filter-sterilized. The pH was adjusted with 1 N KOH to 5.7 before autoclaving. The experimental results were evaluated by Analysis of Variance (ANOVA). Significant differences between the means of embryos calculated per 15 mg initial exptant were evaluated by Duncan's New Multiple Test (Little and Hills 1978). All experiments were repeated at least three times with 10 flasks in each run.

Results and discussion

It has been claimed that development of a system for alfalfa somatic embryogenesis is limited by a number of factors such as initial genotype and explant (Brown and Atanassov 1985; Kao and Michayluk 1981), type and duration of treatment with a morphogenesis inducing factor (Stuart et al. 1985) and composition of the culture medium (Dos Santos et al. 1980). One of the most important steps in developing such a system turned out to be the induction of somatic embryos. It was found that the induction time period strongly affected both the number of embryos obtained and their quality and ability for further development from a single somatic embryo up to a vigorous plant (Stuart and Redenbaugh 1987).

Induction of direct somatic embryogenesis

Direct embryo formation in both selected clones was observed within two weeks (Fig. 5a). Consequently, in order to better assess the effect of the induction term on somatic embryo formation, induction periods of 15, 20, 25 and 30 days were examined. The results showed a significant difference between different induction terms with respect to the total number of embryos detected for M. falcata 47/1-5 (Fig. 1). The largest number of embryos was observed after 25 days of induction. At this time the highest number of torpedo stage embryos was induced. Increase of induction period length after the 15th day appeared to cause the induction of embryos at the cotyledon stage decreasing globular number of embryos.

Several factors that can influence embryo development have been identified: cell density (Halperin 1966), ammonium (Walker and Sato 1981), carbohydrates (Strickland et al. 1987), and osmolarity (Litz 1986). Our results showed that globular embryos induced from both genotypes underwent further development only after cultivation in a medium containing PEG (Fig. 5b). Neither sucrose nor mannitol proved to be effective (data not presented). In order to increase the number of torpedo stage embryos different concentrations of PEG were added to BS/3M medium (Figs. 3 and 4). The largest number of embryos from both genotypes occurred when the medium was supplemented with 2.5% of PEG (p=0.01). When PEG was added to the medium at higher concentrations (5 and 10%) the torpedo stage embryo yield for M. falcata decreased while the total number of embryos was not affected (Fig. 3). The results were found to be a consequence of the increased quantity of embryos at cotyledon stage and/or polyembryos.

Embryo development

Remarkable differences were observed with M. sativa No 2/9R (Fig. 4). First, the total number of embryos decreased along with PEG concentration increase. Second, with a 2.5% PEG concentration the torpedo stage embryo yield was twice as low as in M. falcata. The reverse tendency was detected at the cotyledon stage.