Direct somatic embryogenesis, plant regeneration and in vitro flowering in rapid-cycling Brassica napus

Abstract A simple method to induce somatic embryogenesis from seeds of rapid-cycling Brassica napus is described. Seedlings cultured on Murashige and Skoog (MS) basal medium produced somatic embryos directly on hypocotyls and cotyledons after 2 to 3 subcultures onto the same medium. A low pH of the medium (3.5–5) was more conducive to somatic embryogenesis than a higher pH (6 and 7). Embryogenic potential of the seeds was inversely correlated to seed age: about 41–68% of immature seeds between the ages of 14 and 28 days after pollination (DAP) formed somatic embryos compared to 0–11% of the seeds obtained 29–37 DAP. About 54% of the somatic embryos produced secondary embryos after subculturing onto the same medium. The embryogenic potential of the cultures has been maintained on MS basal medium for 2 years (12 generations) without diminution. Up to 75% of the secondary embryos developed into plantlets on MS medium enriched with 10⁻⁶ M zeatin, and 40% of these produced flowers when transferred to an optimised flower-induction medium. Viable seeds were produced in self-pollinated in vitro flowers.

Keywords Rapid-cycling · Brassica napus · Somatic embryogenesis · Secondary embryogenesis · Regeneration · In vitro Flowering

Abbreviations ABA: Abscisic acid · BAP: 6-Benzyl-aminopurine · DAP: Days after pollination · 2-iP: 6-(γ,γ-dimethallyl-amino)purine · Kinetin: 6-Furfurylaminopurine · MS: Murashige and Skoog · SE₀: Somatic embryo from seed · SE₁: First-generation secondary embryo · SE₂: Second-generation secondary embryo · Zeatin: 6-(4-hydroxy-3-methylbut-trans-2-enylamino)purine

Introduction

Rapid-cycling brassicas, selected from several species of cultivated brassicas, possess many desirable attributes for a model plant system (Wisconsin Fast Plants Manual 1989; Goldman 1999). For example, rapid-cycling lines of brassicas complete their life cycles in about 30–60 days compared to the 6 months to a year required for their cultivated counterparts (Williams and Hill 1986). As such, rapid-cycling brassicas have been used in diverse areas of research and education, such as genetics, molecular biology, plant breeding, cell biology and physiology (Wisconsin Fast Plants Manual 1989; Goldman 1999). Further, rapid-cycling brassicas, which can be readily crossed with cultivated species, can be exploited for brassica improvement programmes as the genetic loci of several useful distinctive morphological, physiological and disease-resistance traits have been clearly mapped and characterised (Williams and Hill 1986; Wisconsin Fast Plants Manual 1989).

While methods to induce somatic and secondary embryogenesis in Brassica napus through anther or pollen cultures have been reported (Thomas et al. 1976; Keller and Armstrong 1977; Lichter 1982; Loh and Ingram 1982; Grubor et al. 1998), there have not been any reports on the induction of direct somatic embryogenesis or continued secondary embryogenesis on plant growth regulator-free medium in B. napus with seeds as the starting material. The occurrence of somatic embryogenesis in the absence of plant growth regulators has been reported in other species, including Clitoria ternatea (Dhanalakshmi and Laksmanan 1992) and Albizzia lebbeck (Gharyal and Maheswari 1981). In Albizzia, somatic embryogenesis occurred directly on the explants (Gharyal and Maheswari 1981).
In this paper, we report a simple method to induce direct somatic embryogenesis from seeds of rapid-cycling *B. napus*. Secondary embryogenesis from the somatic embryos has been maintained for more than 2 years. Plant regeneration and flowering in vitro are also reported here.

**Materials and methods**

**Induction of somatic embryos from seeds**

Seeds of rapid-cycling *Brassica napus* (base population 5-1) were obtained from the Crucifer Genetics Cooperative (CrGC 1997 seed stock). Plants were grown in pots under 16 h of fluorescent light at 25–30°C, and flowers were pollinated at anthesis. Seed age was recorded as number of days after pollination (DAP). Seed pods of different ages were surface-sterilised for 20 min in 20% (v/v) Clorox with two drops of Tween 20 and subsequently rinsed 3 times with autoclaved water. Seeds (10 per dish) were aseptically germinated in 90-mm petri dishes containing 25 ml MS basal medium (Murashige and Skoog 1962) excluding Edamin supplemented with 2% (w/v) sucrose and solidified with Difco agar (0.8% w/v).

**Effect of pH**

The MS basal medium was adjusted to pH 3.5, 4, 5, 6 or 7 prior to the addition of agar and autoclaving at 121°C for 20 min. Both mature seeds (from CrGC) and immature seeds (age range: 15–24 DAP) were used.

**Effect of seed age**

The MS basal medium was adjusted to pH 4 prior to the addition of agar and autoclaving at 121°C for 20 min. Seeds of ages 14–37 DAP were used.

**Production of secondary embryos**

Somatic embryos which emerged from the surface of the seedlings were transferred to fresh MS medium (pH 4) and cultured under the conditions described above. Three somatic embryos were cultured in each 90-mm petri dish, and the secondary embryos that developed from the somatic embryos were propagated by subculturing onto fresh medium every month.

**Plantlet regeneration and in vitro flowering**

Batches of 15–32 secondary embryos (1–3 mm long) were cultured individually in 60-mm petri dishes containing 10 ml MS medium (pH adjusted to 5.8 before the addition of 0.8% w/v Difco agar) supplemented with 10–2–10–4 M kinetin, BAP, 2-iP or zeatin for 4 weeks. Secondary embryos with two or more normal leaves were considered to have regenerated into plantlets. These plantlets were transferred to 30 ml MS basal medium with or without nitrogen, in GA7 (Magenta Corp, USA) culture containers, for further growth and development.

In order to improve flowering, we cultured the secondary embryos on MS medium with 10–5 M BAP for 2 weeks and then transferred them to tentative flower-induction media consisting of modified MS medium. The modifications included various combinations of changes in nitrogen, phosphate and sucrose concentrations, and the addition of BAP, zeatin or iP (Table 5). Flower development was assessed 6 weeks after the secondary embryos were transferred to the flower-induction medium. Pollen viability was assessed using an aceto-carmine staining method (Evans and Reed 1981).

**Statistical analysis**

The results were analysed using one-way ANOVA and Tukey’s multiple comparison test (5% level). Pooled values were used for results from experiments repeated once.

**Results and discussion**

**Induction of somatic embryogenesis in seeds**

Many of the seedlings developed swollen hypocotyls within 2 weeks after germination. Of these, the majority produced somatic embryos (SE0) directly on the hypocotyls and/or cotyledons (Fig. 1a) after 2 to 3 subcultures – i.e. by the second or third month – although about 1% produced SE0 within 3 weeks after culture. The average number of SE0 produced per embryogenic seedling from the immature seeds was 6.4±1.0, although 78 SE0 per seedling has been observed. The number of SE0 produced per explant was not significantly affected by the pH of the medium (Table 1). However, with respect to the percentage of explants producing SE0, low medium pH (3.5–5) was more conducive to somatic embryogenesis than higher pH (6–7) (Table 1). Seedlings from the matured seeds produced somatic embryos only when the pH of the medium was 4; immature seeds (15–24 DAP) underwent somatic embryogenesis in all of the media tested, with the highest yield (43.6%) at pH 3.5 (Table 1). The effect of medium pH observed here contrasts with that observed for carrot cultures, where a low medium pH was found to be detrimental to somatic embryo induction; somatic embryogenesis was reduced at pH 4.5 and completely inhibited at pH 4, while a relatively higher medium pH of 5.7 allowed more zygotic embryos to produce somatic embryos (Smith and Krikorian 1990). A low medium pH – below 4.5 – has been reported to disrupt the sensitive internal regulation of cytoplasmic pH in several species of green alga and in mung bean root-tip cells, so that a net influx of H+ results in a drop in cytoplasmic pH (Mimocha 1987). Auxin has been found to be essential for somatic embryo induction in many plant species (Merkle et al. 1995). Also, one of the chief characteristic primary cellular responses to auxin is a decrease in the cytosolic pH, which in turn may modulate cytosolic free calcium (Dudits et al. 1995), an important secondary messenger in plant cells. Thus, it is possible that low external pH may simulate auxin action during the induction of