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PLANT REGENERATION FROM COTYLEDON TISSUES OF COMMON BUCKWHEAT (*FAGOPYRUM ESCULENTUM* MOENCH)

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Summary

Plants were regenerated from cotyledon tissue of greenhouse grown seedlings of common buckwheat (*Fagopyrum esculentum* Moench.). Maximum callus regeneration was induced on Murashige and Skoog (MS) medium containing 2,4-D (2.0 mg l^{-1}) and kinetin (KIN) (0.2 mg l^{-1}) and either 3 or 6% sucrose. Friable callus was transferred to MS media containing KIN and benzylaminopurine (BAP) at varied concentrations for embryogenic callus induction. The optimum medium for embryogenic callus induction was found to be MS medium supplemented with 0.2 mg l^{-1} KIN, 2.0 mg l^{-1} BAP and 3% (w/v) sucrose. Variation of sucrose from 3 to 6% did not show any significant effect on callus induction or embryogenesis. Regeneration of embryonic callus varied from 13 to 32%. Whole plants were obtained at high frequencies when the embryogenic calluses with somatic embryos and organized shoot primordia were transferred to half-strength MS media with 3% sucrose. Regenerated plants after acclimation were transferred to greenhouse conditions, and both vegetative and floral characteristics were observed for variation. This regeneration system may be valuable for genetic transformation and cell selection in common buckwheat.

Key words: common buckwheat; *Fagopyrum esculentum*; cotyledon explant; plant regeneration; somatic embryogenesis.

Introduction

At present, information about tissue culture in buckwheat is limited and is mainly restricted to micropropagation. *Fagopyrum* species are dipliod ($2n = 16$), but tetraploid varieties either occur spontaneously or can be induced. Buckwheat has for centuries remained a crop with low seed set due to certain characteristics which prevent the application of conventional breeding methods (Kreft, 1983). The main obstacles in buckwheat breeding include its very strong self/cross-incompatibility and its indeterminate type of growth and flowering.

Modern biotechnology may provide means to address these problems in a novel way (Neskovic et al., 1995). *In vitro* regeneration of buckwheat has been reported from explants such as hypocotyls (Yamane, 1974; Lachmann and Adachi, 1990); cotyledons (Srejovic and Neskovic, 1981; Miljus-Djukic et al., 1992); immature inflorescence (Takahata, 1988); and anthers (Adachi et al., 1989; Bohanec et al., 1993).

This study of somatic embryogenesis and plant regeneration of callus cultures from cotyledon segments of the cultivated buckwheat species *Fagopyrum esculentum* differs from existing studies in the growth regulator combinations used. Somatic embryogenesis has previously been reported in cultures of immature embryos of common buckwheat (Neskovic et al., 1987; Rumyantseva et al., 1989) and of Tartary buckwheat (Rumyantseva et al., 1989; Lachmann and Adachi, 1990). The objective of this research was to develop a plant regeneration system for common buckwheat for future application of genetic transformation.

Materials and Methods

Mature dehulled seeds of cross-pollinated *F. esculentum* cv. Miyazaki zairai plants were treated with a 20 s immersion in 70% (v/v) ethanol followed by 30 min in 0.3% (v/v) sodium hypochlorite solution with drops of detergent (Tween 80). After three rinses with autoclaved water, the seeds were placed on moistened sterile filter papers in petri dishes and incubated in the dark at 25°C for 48 h.

After germination of seeds, the cotyledons were excised and placed on callus induction medium. Twelve combinations, with varied concentrations of auxin and cytokinins, were tested for callus induction efficiency. Approximately equal numbers of explants were placed on medium containing MS (Murashige and Skoog, 1962) supplemented with myoinositol (100 mg l^{-1}), sucrose (3 or 6%) and different concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D), α-naphthalene acetic acid (NAA) and kinetin (KIN). The pH was adjusted to 5.7 before autoclaving, and the media were solidified with 0.8% (w/v) agar. As they developed, the somatic embryos and miniature organogenic shoots were transferred to half-strength MS hormone-free medium with 3% sucrose for rooting and further growth.

The culture conditions were maintained at 16/8 h (light/dark) photoperiod at 25°C. Plants with a well developed root system were transplanted to an organic soil mixture for hardening under growth-chamber conditions (16/8 h light/dark) photoperiod; at 25°C (80% humidity) for 2 wk; and later

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TABLE 1

CALLUS INDUCTION EFFICIENCY IN *FAGOPYRUM ESCULENTUM*

Hormone combination (mg l^{-1})	No. of cotyledon segments plated	Callus initiation (%)
(1) MS + KIN(0.2) + 2, 4-D(1.0) + 3% sucrose	30	100
(2) MS + KIN(0.2) + 2, 4-D(2.0) + 3% sucrose	30	100
(3) MS + KIN(0.2) + 2, 4-D(3.0) + 3% sucrose	30	100
(4) MS + KIN(0.2) + NAA(0.1) + 3% sucrose	30	0
(5) MS + KIN(0.2) + NAA(0.3) + 3% sucrose	30	0
(6) MS + KIN(0.2) + NAA(0.5) + 3% sucrose	30	0
(7) MS + KIN(0.2) + 2, 4-D(1.0) + 6% sucrose	30	100
(8) MS + KIN(0.2) + 2, 4-D(2.0) + 6% sucrose	30	100
(9) MS + KIN(0.2) + 2, 4-D(3.0) + 6% sucrose	30	100
(10) MS + KIN(0.2) + NAA(0.1) + 6% sucrose	30	0
(11) MS + KIN(0.2) + NAA(0.3) + 6% sucrose	30	0
(12) MS + KIN(0.2) + NAA(0.5) + 6% sucrose	30	0

transferred to regular greenhouse conditions. Data were collected for phenotypic characteristics, both vegetative and floral.

Results

Of the various media tested, MS media supplemented with KIN and 2,4-D were found to be suitable for callus induction and proliferation of buckwheat cotyledon tissue. Prolonged maintenance of up to 4 wk resulted in root formation. The optimum callus induction of 100% (Table 1) with minimum root induction was obtained on MS media supplemented with KIN (0.2 mg l^{-1}), 2,4-D (2.0 mg l^{-1}) and either 3 or 6% sucrose. Each subculture was carried out on this initiation medium for 3 wk before being transferred onto embryogenic callus induction medium. After callus induction, the subcultures were carried out on MS medium containing 0.2 mg l^{-1} KIN and benzylaminopurine (BAP) at varied concentrations. The sucrose concentration was maintained at 3%. After two passages in the same media with an interval of 3 wk, KIN at 0.2 mg l^{-1} and BAP at 2.0 mg l^{-1} produced embryogenesis at a frequency of 32% (Table 2; Fig. 1A,B). Increased or decreased levels of BAP had lower frequencies of embryogenic initiation. The callus, other than the embryogenic region, turned brown, presumably due to excess exudation of phenolics.

To maintain the embryogenic potential, embryogenic tissues were separated from the underlying translucent dark callus while subculturing onto fresh medium. The mean number of embryogenic calluses produced after incubation ranged from 13 to 32% in the different media combinations.

TABLE 2

EFFECT OF BAP AND KIN ON EMBRYOGENIC CALLI INDUCTION IN *FAGOPYRUM ESCULENTUM*

Hormone concentration			
BAP	KIN	No. of nodular calluses plated	Mean number of embryogenic calluses
0.5	0.2	30	0.13ac
1.0	0.2	30	0.15ab
2.0	0.2	30	0.32cd
3.0	0.2	30	0.25acd
5.0	0.2	30	0.26ad

MS medium with 3% sucrose was used as basal media. Five replicates were maintained for each treatment. Mean values with the same letter are not significant (protected LSD, $P = 0.05$).

All the treatments were maintained under diffused light conditions. The somatic embryoids were then transferred to hormone-free half-strength MS medium containing 3% sucrose. The embryos developed into plants (Fig. 1C). The frequency of plant regeneration varied with respect to the embryogenic callus induction media. The highest number of plants was obtained when the embryogenic callus induction medium was MS supplemented with 2.0 mg l^{-1} BAP and 0.2 mg l^{-1} KIN and 3% sucrose. After 3 wk in hormone-free media, well rooted plantlets were separated, washed and transplanted to an organic soil mixture in small cups. After acclimation, the hardened plants were then transferred to large pots under greenhouse conditions (Fig. 1D). The regenerated plants were found to vary in their leaf morphology. Although flowering was normal, the flower size (diameter) varied from 5–6 mm to 7–8 mm in regenerated plants. Seed obtained after cross-pollination matured within 101 d. Morphological differences in seed characters were also observed (Fig. 1E).

Discussion

Regeneration of *F. esculentum* was achieved on media containing different growth regulator combinations. This occurred with relatively high frequency (32%) on media containing BAP (2.0 mg l^{-1}) and KIN (0.2 mg l^{-1}). The somatic embryos developed in clusters on the embryogenic callus. At the cotyledonary stage, many embryos were small and not very conspicuous. A similar phenomenon was reported by Neskovic et al. (1995). However, in our experiments with *F. esculentum* direct regeneration of shoots was also observed in a few cases. Variations in leaf morphology, flower size, seed morphology and size occurred among the regenerated plants. The reasons for this observation were not determined. The effect of sucrose remains somewhat obscure, as the sucrose content in callus induction and later regeneration media had no significant effect on the development of somatic embryos. This is in contrast to the findings of Neskovic et al. (1987) and Lachmann (1991) who reported that the sucrose content was critical for the development of somatic embryos in buckwheat. Species specificity of buckwheat could be one of the explanations. While *F. esculentum* is neutral with respect to sucrose, other species of *Fagopyrum* may be sucrose-sensitive. Shoot regeneration may also

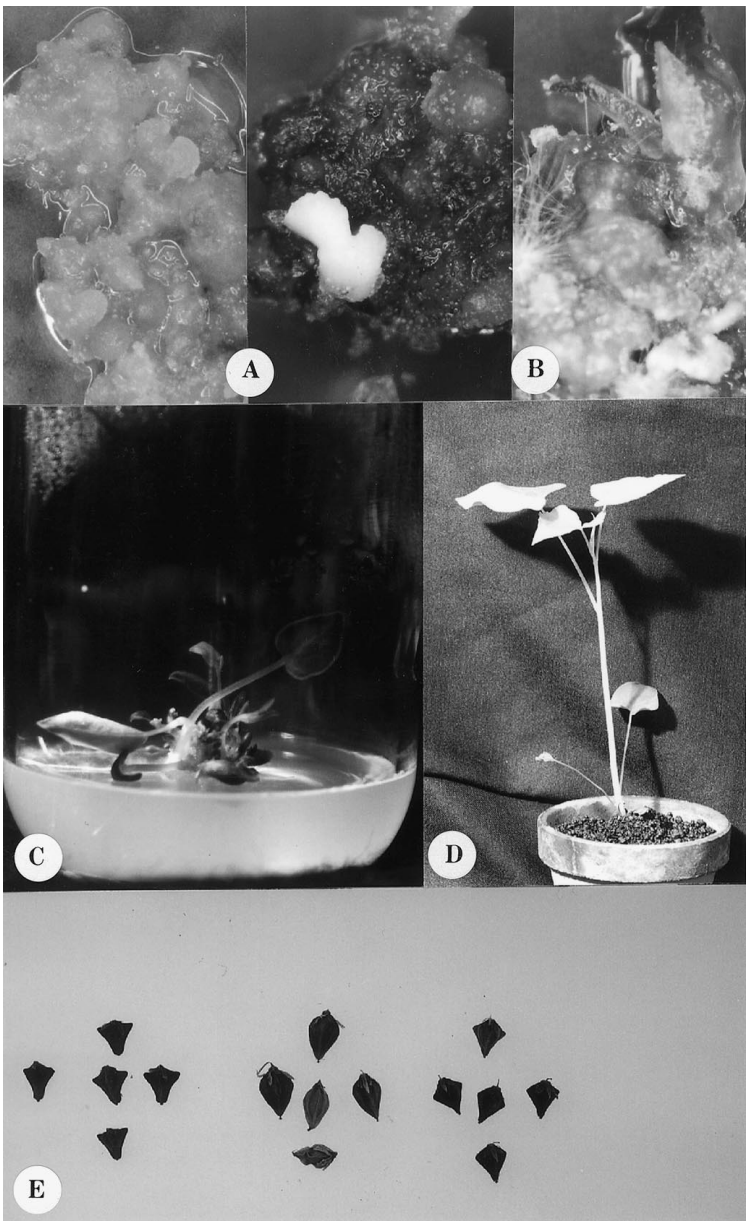

FIG. 1. Plant regeneration of common buckwheat (*Fagopyrum esculentum* Moench.) in culture media. (A) Embryogenic callus formed from cotyledons on MS media supplemented with 2.0 mg l^{-1} BAP and 0.2 mg l^{-1} KIN; (B) somatic embryo formation; (C) regeneration of plants on hormone-free media; (D) regenerated plants after hardening; (E) seeds from regenerated plants.

vary depending on the genotype of the donor plant. Differences in shoot regeneration have been found in many buckwheat species (Lachmann and Adachi, 1990; Neskovic et al., 1995). This variation was also found in our experiments. As explants were isolated from a mixed seedling population, all segments of one leaf may have formed shoots, whereas all segments of another leaf might have been incapable of forming shoots, even in the same petri plate. Explants from all donor plants formed shoots, but the regeneration frequency and the number of shoots regenerated varied widely among the seedlings.

These experiments demonstrate that plant regeneration from cotyledons of common buckwheat can be obtained readily and reliably at a moderate frequency. This regeneration system will be valuable for genetic transformation and cell selection of common buckwheat.

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