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An efficient protocol for shoot organogenesis and plant regeneration of buckwheat (*Fagopyrum esculentum* Moench.)

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Abstract

The establishment of a simple and efficient protocol for shoot organogenesis and plant regeneration from the lateral cotyledonary meristems of buckwheat (Fagopyrum esculentum Moench.) is reported. We investigated the effect of different concentrations of cytokinins (BAP, kinetin and TDZ) on the efficiency of shoot organogenesis in buckwheat. Treatment with BAP significantly induced shoot regeneration from cotyledon cultures of buckwheat. The highest number of shoots per explant (5.7) and shoot length (1.3 cm) were obtained on MS medium containing 4.0 mg/l BAP. The addition of the $AgNO_3$ at an optimal concentration of 7 mg/l substantially improved the shoot regeneration frequency, which was around 30% more than in control, but declined at concentrations beyond the optimal one. The regenerated shoots (about 1 cm in length) displayed a normal morphology and could be easily rooted without any plant hormone treatment. The rooted plants were hardened and transferred to soil with a 72% survival rate, where they grew normally.

Keywords: buckwheat, cotyledonary meristem, *Fagopyrum esculentum* Moench., plant regeneration, shoot organogeneis

Introduction

Buckwheat, *Fagopyrum esculentum* Moench, is an agronomic species of the Polygonaceae family that belongs to the genus *Fagopyrum*. It has been proven to be widely adapted in North America and Europe. The crop matures in a relatively short period of time (70 to 80 days) and makes an excellent rotation with cereal grains [17; 2]. Buckwheat seeds are used for human food material and as a potentially important source of rutin [5; 6]. From previous research findings, it is well known that rutin, a flavonol glycoside, has strong antioxidant effect and possesses other interesting pharmacological properties such as anti-inflammatory, anticarcinogenic, antithrombotic, cytoprotective and vasoprotective activities [7; 10].

The regeneration of buckwheat plants via organogenesis or somatic embryogenesis from cotyledon explants has been previously reported [14; 11; 16]. However, the procedure and efficiency of buckwheat plant regeneration was complicated and even not easily available to the scientists yet. A simple and efficient method for the regeneration of autonomous plants from tissue culture is essential to establish a genetic transformation protocol for *F. esculentum*. In this paper, we report the development of a rapid and simple method for high-

efficiency plant regeneration from the lateral cotyledonal meristems of buckwheat, *F. esculentum*.

Materials and Methods

Plant materials

Dehulled seeds of *Fagopyrum esculentum* were surface-sterilized with 70% (v/v) ethanol for 30 sec immersion, washed in 1% sodium hypochlorite solution containing a few drops of Tween 20 for 20 min and followed by three washes of sterilized water. Seeds were placed on the medium in Petri-dishes (100 x 15 mm) that contained approximately 25ml of culture medium. Six seeds were cultured in each Petri dish. The basal medium consisted of salts and vitamins of MS (Murashige & Skoog) [12] medium and solidified with 0.3% (w/v) Gelrite. The pH of media was adjusted to 5.8 before adding gelling agents. The media were autoclaved at 1.1 kg cm^{-2} (121 °C) for 20 min. The seeds were germinated at 25±1 °C in a growth chamber with a 16-h photoperiod under standard cool white fluorescent tubes (35 $\mu mol\ s^{-1}\ m^{-2}$).

Shoot organogenesis

Cotyledons of seven-day-old seedlings were detached from the embryonic axis, by removing the whole cotyledonal node. Excised cotyledon pairs were then separated and the apical meristem removed. Individual cotyledons were placed with adaxial side uppermost on media. Five excised cotyledons were cultured in (100 x 25 mm) Petri dishes on Gelrite solidified MS basal medium supplemented with BAP, Kinetin, and TDZ (1- phenyl-3-(1,2,3-thiadiazol-5-yl) urea = thidiazuron) at concentrations of 0.0, 0.5, 1.0, 2.0, 4.0, 6.0 mg/l for shoot regeneration during 6 weeks, being transferred to fresh medium every 2 weeks. For improvement of shoot regeneration, the different concentrations of silver nitrate (0, 1, 3, 7, 10, 20 mg/l $AgNO_3$) were tested in MS medium supplemented with 4.0 mg/l BAP (6-benzylaminopurine).

Rooting of regenerated shoots

Microshoots (around 1 cm long) were placed on MS medium without growth regulators. The medium was solidified with 3g/l Gelrite and dispensed at 70 ml per Magenta box. Four shoots were cultured in each box and were incubated at 25±1 °C in a growth chamber with a 16-h photoperiod, under standard cool white fluorescent tubes (35 $\mu mol\ s^{-1}\ m^{-2}$) for 50 days. After 50 days, shoots were washed with sterile water to remove Gelrite from their roots, transferred to the pots containing autoclaved vermiculite, covered with polythene bags to maintain high humidity and kept at 25±1°C in a growth chamber for 1 week. After 1 week the bag were perforated. These plants were then transferred to the green house.

Results

A simple and effective protocol has been developed for the *in vitro* plant regeneration of buckwheat, *F. esculentum*. For establishing a plant regeneration protocol, we investigated the effect of different concentrations of cytokinins (BAP, kinetin and TDZ) on the efficiency of shoot organogenesis in buckwheat. The treatment with BAP significantly induced shoot regeneration from cotyledon explants of buckwheat. Kinetin also promotes shoot regeneration

of buckwheat but its impact was much lower than that of BAP. On the other hand TDZ did not properly induce shoot regeneration from cotyledonal explants of buckwheat.

With the increase of BAP concentration up to 4 mg/l the regeneration rate, the number of shoots and shoot length also increased, but beyond this concentration of BAP all these parameters decreased (Table 1). The highest number of shoots per explant (5.7) and shoot length (1.3 cm) were obtained on MS medium containing 4.0 mg/l BAP (Table. 1).

Table 1. Effect of different concentrations of BAP and Kinetin on shoot regeneration from cotyledonal explants of *Fagopyrum esculentum* Moench after 6 weeks of culture on MS medium.

Cytokinin	(mg/l)	Regeneration rate* (%)	No. of shoots /explants*	Shoot length** (cm)
BAP	0.5	35	0.8±0.2	0.8±0.1
	1.0	57	2.3±0.3	1.0±0.1
	2.0	64	4.5±0.7	1.2±0.1
	4.0	72	5.7±0.8	1.3±0.1
	6.0	63	2.8±0.7	1.0±0.1
Kinetin	0.5	0	--	--
	1.0	38	1.6±0.2	0.8±0.1
	2.0	53	2.6±0.3	1.1±0.1
	4.0	61	3.7±0.4	1.1±0.1
	6.0	47	2.1±0.2	0.9±0.1

-- No response

* From 100 cotyledonary explants tested.

** Values represent the mean ± standard deviation of 50 shoots

When buckwheat cotyledons were cultured on MS solid media supplemented with 4mg/l BAP, various stages of the *F. esculentum* shoot organogenesis process were shown (Figure 1). During the initial stage (1-2 weeks of incubation), there was some expansion and proliferation of cells at the cut surface but callus growth was limited (Fig. 1-A). After that the cut end of the cotyledonary explant enlarged and within 3 weeks shoot primordia and small elongated shoots had formed adjacent to the cut surface (Figure 1-B). We observed that cells of the epidermis proliferated to produce shoots directly, without an intervening callus phase. The regenerated shoots were developed from shoot primordia within 3-4 weeks. After six weeks of culture, an average of 5 to 6 fully developed shoot of at least 1.3 cm in length were produced supposedly from cotyledonal explants (Figure 1-C).

The addition of the $AgNO_3$ up to a level of 10 mg/l substantially improved the shoot regeneration frequency. However the treatment with 20 mg/l $AgNO_3$ inhibited the shoot regeneration compared to control (Table 2). The concentration of 7 mg/l $AgNO_3$ was the optimum to induce the highest shoot regenerating explants (87%), the highest number of shoots per explants (7.9) as well as the highest shoot growth (1.8 cm). The widespread effectiveness of ethylene inhibitors, such as $AgNO_3$, at promoting shoot organogenesis in several plant species has been reviewed by Kumar et al. [8]. The regenerated shoots (1 cm in length) had a normal morphology and could be easily rooted without any plant hormone treatment. Roots formed on shoots within 4 weeks in Magenta box containing solid MS hormone free media. The rooted plants were hardened and transferred to soil with a 72% survival rate, where they grew normally.

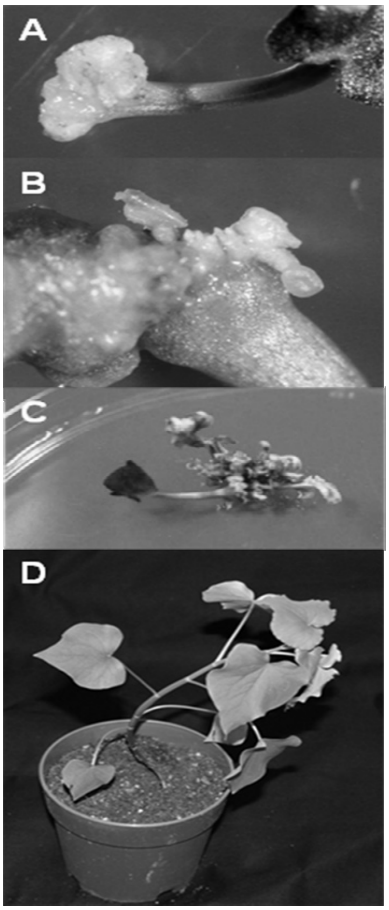

Fig. 1. Shoot organogenesis in buckwheat, *Fagopyrum esculentum*.
A) The expansion and proliferation of cells at the cut surface of cotyledonal explants one to two weeks after culture initiation (x 10);
B) Shoot primordia emerging from a cotyledonary explant of *F. esculentum* three weeks after the cultivation on MS solid media supplemented with 4mg/l BAP (x 15).
C) After six weeks of culture, fully developed shoots were produced from cotyledonal explants (x 1.5).
D) Regenerated plant in the pot one month after the cultivation in the growth chamber (x 0.2).

Table 2. Effect of different concentration of $AgNO_3$ on shoot regeneration from cotyledonary explants of *Fagopyrum esculentum* Moench after 6 weeks on shoot regeneration medium consisting in MS salts and vitamins, 30 g/l sucrose, and 4 mg/l BAP.

$AgNO_3$ (mg/l)	Regeneration rate* (%)	No. of shoots /explants*	Shoot length** (cm)
0	72	5.7±0.8	1.3±0.1
1	74	5.9±0.7	1.3±0.1
3	78	6.1±0.9	1.5±0.2
7	87	7.9±1.2	1.8±0.2
10	85	7.6±1.1	1.6±0.2
20	67	5.3±1.0	1.2±0.1

* From 100 leaf explants tested.
** Values represent the mean ± standard deviation of 50 shoots.

Discussion

Plant tissue culture plays an important role in plant biotechnology. Plant regeneration protocols are an essential part of plant genetic transformation leading to plant improvement. More recently, the term 'regeneration' has been broadly used in the context of tissue culture as the production of whole plants from cells, tissues, organs, meristems or zygotic embryos cultivated *in vitro*. There are two major systems of plant regeneration, organogenesis and somatic embryogenesis. These systems are defined based on the developmental pathways through which a whole plant is regenerated.

In vitro regeneration of buckwheat *via* somatic embryogenesis or shoot organogenesis has been reported from various explants such as hypocotyls [18; 9; 4], cotyledons [14; 11; 16], immature inflorescence [15], immature embryos [13] and anthers [1; 3]. Our protocol for the plant regeneration of buckwheat using the lateral cotyledonal meristems is simple, efficient and rapid in comparison with other reports that describe the organogenesis or somatic embryogenesis processes in buckwheat by using various explants.

Conclusions

From our a little effort to establish an efficient protocol for shoot organogenesis and plant regeneration from the lateral cotyledonary meristems of buckwheat (*Fagopyrum esculentum* Moench.), the production of buckwheat regenerated plants could be used in a possible transformation protocol which might create new opportunities to study the molecular and metabolic regulation of producing useful secondary metabolites in *F. esculentum.*

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