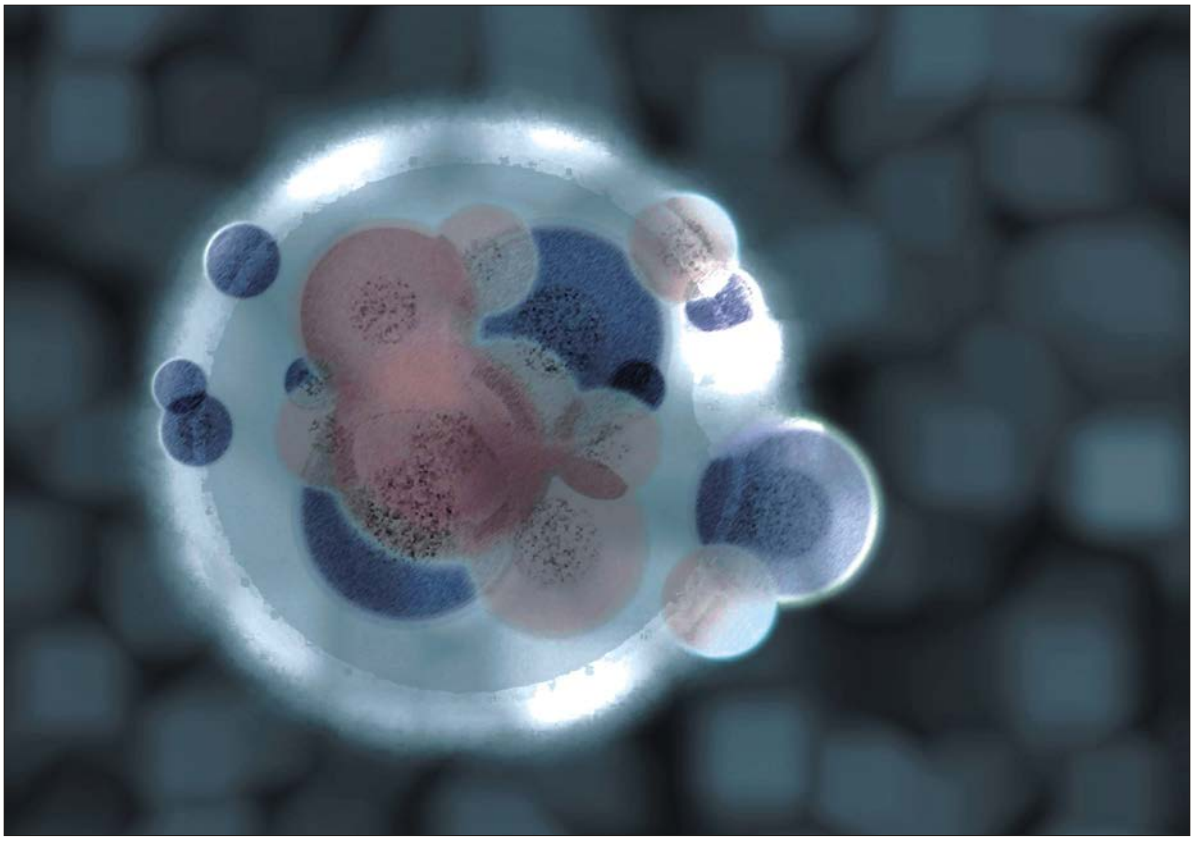

Journal of Biological Sciences

ISSN 1727-3048

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Journal of Biological Sciences 9 (1): 1-11, 2009
ISSN 1727-3048

An *in vitro* Ovule Culture Technique for Producing Interspecific Hybrid Between Tartary Buckwheat and Common Buckwheat

[1]M. Asaduzzaman, [2]M. Minami, [2]K. Matsushima and [2]K. Nemoto
[1]The United Graduate School of Agricultural Science,
Gifu University (Shinshu University), Nagano 399-4511, Japan
[2]Graduate School of Agriculture, Shinshu University, Nagano 399-4511, Japan

Abstract: An interspecific hybrid of the *Fagopyrum* species *F. tataricum* cv. CT-1 (2x and 4x) and *F. esculentum* cv. Botansoba (2x) and GreatRuby (4x) was obtained through *in vitro* ovule culture. Seven to ten days old ovules were excised and cultured on 1/2 MS and MS media and rescued ovules were subjected to different combinations and concentrations of BA, NAA, IAA and zeatin added individually and in various combinations along with sucrose. Effects of the hormones were used to evaluate their potentialities for direct regenerations, callus induction and callus regeneration through ovule culture in interspecific cross between tartary and common buckwheat. Interspecific hybridization in *Fagopyrum* used ovule culture two methods development in this study. The result showed that no single medium was adequate to ensure complete development of the fertilized ovules to plantlets, thus necessitating a sequential three step transfer to different media. RAPD-PCR analysis confirmed hybridity of the regenerated plant.

Key words: *Fagopyrum*, diploid, tetraploid, hormones, culture medium, RAPD

INTRODUCTION

The buckwheat species is of the genus *Fagopyrum* and one of the main species of this genus is *F. esculentum. F. esculentum* is not only used as a human food source but common buckwheat is also characterized by its seed's high protein content, as well as the medicinal value derived from its rutin content, which is important for human health and for treating human diseases (Marshall and Pomeranz, 1982; Koh *et al*., 1988; Choi *et al*., 1988; Cheng and Ni, 1992). Due to rice over-production, cultivation of common buckwheat (*F. esculentum*) as an alternative crop has been increasing in Japan. However, productivity of common buckwheat has been low with an unstable yield. Thus, buckwheat has not emerged as an economical crop; the dimorphic self incompatibility system, limited variation in its reproductive system and susceptibility to different pests and diseases being the major yield limiting factors (Campbell, 2003; Nair *et al*., 1999; Marshall, 1969; Kreft, 1983; Guan and Adachi, 1992).

Fagopyrum tataricum cultivated buckwheat species grows in the high-altitude mountain areas of Asia. On the other hand, *F. tataricum* usually called tartary buckwheat is a species which is self-compatible with homomorphic flowers and has a high yield, self-fertility and probable tolerance to frost but its seeds lack the desired flavor (Adachi, 1986; Ruszkowshi, 1980; Baniya *et al*., 1992).

One approach which may lead to increased seed yield is to cross common buckwheat with tartary buckwheat which is self-compatible. It has been reported that some characteristics (such as leaf area, rate of photosynthesis and root system) of tartary buckwheat are very similar to those of common buckwheat and that tartary seed yield is three times higher than with common buckwheat (Adachi, 1986; Ruszkowski, 1980). To overcome the complexities associated with this crop, breeders around the world are now initiating several strategic researches. One of the strategies for buckwheat improvement includes wide hybridization of cultivated buckwheat with its closely related wild relative's species.

Conventional breeding techniques have not been successful in hybridizing common buckwheat with tartary (Morris, 1951; Ruszkowski, 1980; Adachi *et al*., 1989; Samimy, 1991). So far, application of protoplast fusion has also failed because the resulting Calli did not regenerate (Lachman, 1991). Ujihara *et al*. (1990) have successfully hybridized tetraploid wild buckwheat (*F. cymosum*, 2n = 32) with tetraploid common buckwheat using ovule culture. Efforts to improve common buckwheat have been made through interspecific

Corresponding Author: M. Asaduzzaman, The United Graduate School of Agricultural Science,
Gifu University (Shinshu University), Nagano 399-4511, Japan

hybridization between common buckwheat and related species *F. cymosum* (Ujihara *et al*., 1990; Suvoeova *et al*., 1994; Hirose *et al*., 1995; Woo *et al*., 1999) and *F. tataricum* (Morris, 1951; Samimy, 1991; Hirose *et al*., 1995; Samimy *et al*., 1996; Chen, 1998; Wang and Campbell, 1995; Fesenko *et al*., 2001; Wang *et al*., 2002). However, success has been limited due to sterility of the hybrids. The first successful hybridization between *F. esculentum* and *F. homotropicum* was reported by Campbell (1995) in a crop-improvement program designed to produce self-pollinated buckwheat.

Common buckwheat (*F. esculentum*) is widely cultivated but shows low yielding ability due to self-incompatibility. To introduce self-compatibility into common buckwheat, interspecific crosses have been carried out between common buckwheat and self-compatible *Fagopyrum* species. Interspecific hybridization of common buckwheat with *F. homotropicum*, a wild self-compatible species, was successful and self-compatibility was introduced into common buckwheat, but undesirable traits such as small seed size and seed shattering habit were also introduced from *F. homotropicum*. Tartary buckwheat is a cultivated self-compatible species with medium seed size and non-seed shattering, so tartary buckwheat is better as a source of self-compatibility than *F. homotropicum*. Hybrid plants between common buckwheat and tartary buckwheat had been produced using ovule culture, but hybrid plants were viable only *in vitro* and sterile. Therefore, a wide genetic base is the fundamental requirement in any crop-improvement programme. *In vitro* ovules culture methods could be used to speed up the breeding programme. Using ovule culture, we obtained hybrid plants which can grow in field and are fertile. In this aspect ovule culture has a very important role, because *in vitro* culture of ovule in interspecific hybridization is one of the desirable methods for promotion of breeding in genus *Fagopyrum*. The present study was undertaken transfer to desirable traits from hybrids to development and improvement of ovule culture rapid methods by using *in vitro* ovule culture techniques in buckwheat. In this study we describe the results of the effect of growth regulators on hybrid plant direct regeneration, as well as callus induction and callus regeneration through ovule culture in interspecific cross between tartary buckwheat and common buckwheat. Hybridity was certified conventional RAPD analysis.

MATERIALS AND METHODS

The species used for the interspecific crosses were *F. tataricum* cv. CT-1 (2x, 4x) and *F. esculentum* cv. Botansoba (2x) and cv. GreatRuby (4x) as female and male parents, respectively. These varieties were grown in the pot and all crosses were made by hand pollination after flowering. Young hybridized ovules are rescued using ovule culture. This experiment was conducted from October 2005-September 2008 in Shinshu University, Japan.

Crossing: The female parent *F. tataricum* self compatible homostylic flowers were emasculated one day before crossing. Male parent *F. esculentum* long pin type flower pollen was transferred on the stigma of *F. tataricum*. After emasculation, the emasculated flowers were isolated from other flowers and developing seeds. Emasculation was performed in the early morning-about 8-9 am and crossing was done between am 9 am to 12 pm. The crossed flowers were bagged for two or three days following pollination.

Media preparation: Culture media for the growth of ovule culture consist of various inorganic and organic nutrients. The inorganic nutrients were supplied as water-soluble compounds. Organic nutrients included carbon source and were usually supplied with sucrose as glucose and organic growth substances, mainly vitamins. The medium mixed with fundamental inorganic and organic nutrients is called the basic medium. In order to regulate the growth and differentiation of the cultured cells, it is always necessary to supplement the basic medium with various physiologically active substances-phytohormones such as auxins and cytokinins. The basic medium not only supplies nutrients but also provides suitable pH and osmotic pressure conditions for the growth of culture.

1/2 MS and MS (Murashige and Skoog, 1962) media and the various combinations of growth regulators in addition to the basic media were used in ovule culture studies. These media were solidified with 0.7% purified agar. The pH was adjusted to 5.7-5.8 with 1N HCl or 1N KOH prior to sterilization and the pH was always adjusted before the addition of agar. All ovule culture work was carried out in a sterilized environment on a laminar airflow cabinet.

***In vitro* ovule rescue:** For ovule culture, ovaries are better enlarged 7-10 days after pollination. The enlarged ovaries from the crosses were collected. The ovaries were surface sterilized with 70% (v/v) ethanol for 30 sec and 2% commercial bleach containing sodium hypochloride (NaOCl) for 10 min. Ovaries were then thoroughly washed with autoclaved distilled water several times, after which they were carefully excised under microscope and plated (1 ovule per tube) in test tubes which

contained 10 mL 1/2 MS and MS media with the addition of different concentrations and combinations of BA, NAA, IAA, zeatin and 2 and 3% sucrose. We used two methods to regenerate to plantlets: One was developing directly into plantlets, while the other required the use of three culture media after plantlet development was completed, inducing media for callus induction, regeneration media for shoot development and rooting media for root formation. The ovule culture were maintained at 22±2°C in 16 h of light and an 8 h dark period.

Acclimation: Fully developed plantlets were removed from the test tube and washed in distilled water. Plantlets were then transferred to sterilized vermiculite and grown for about 2 weeks in *in vitro* condition. Afterward plantlets were transferred into potted soil and grown in the greenhouse.

DNA extraction and PCR analysis: Genomic DNA was isolated from young and fresh leaves (0.30 g sample) using Automatic Nucleic Acid Extractor (Quick Gene-810, FUJI FILM, Japan).

After template DNA was isolated from the parents and the F_1 hybrids, 10-mer arbitrary primer (5´-3´) OPK15 (CTCCTGCCAA) prepared by (Operon Technologies, CA, USA) was used for PCR amplification. Each PCR reaction included 12 µL volume containing 1×buffer (supplied by Takara, Shiga, Japan), 200 µM each of dNTPs, 0.42 µM arbitrary primer, 0.5 units of *Taq* DNA polymerase (Takara, Shiga, Japan) and 12 ng template DNA. The DNA PCR amplifications were performed with a BIO-RAD i Cycler (Japan) as follow: one step of 3 min at 94°C, then 40 cycles of 1 min at 94°C, 2 min at 40°C, 2 min at 72°C and a final step of 5 min at 72°C.

The amplification products were separated by electrophoresis in 1.7% agarose gels stained by 10 µL ethidium bromide, 2 µL loading buffer was added to each PCR reaction and 7 µL of each reaction was loaded on the gel. The DNA marker in each gel was used as the check. Electrophoresis was set at 100v for 1 h. The electrophoresed gel was photographed using UV transilluminator after staining with ethidium bromide.

RESULTS AND DISCUSSION

Two species of buckwheat, both in diploid and tetraploid levels, were crossed together and their ovules were rescued after fertilization. Rescued ovules were subjected to different concentrations of hormones to evaluate their potentialities for direct regeneration, callus induction and callus regeneration. For hormonal treatments, 1/2 MS and MS media were used as a basal medium and they were supplemented with different concentrations of NAA, BA, IAA, 2, 4-D and zeatin. Two methods were practiced to regenerate the plantlets in this experiment.

Interspecific hybridization between Tartary×Common buckwheat using direct method: In case of direct regenerations, the ovules rescued from the cross of diploid species were treated with different concentrations and combinations of NAA, IAA, BA and zeatin. Data were collected on the number of ovules rescued, number of embryo emerged, percentage of emerged embryos and number of surviving hybrid and these results are shown in Table 1. For regeneration, 10 to 16 rescued ovules were treated, but only 1 to 4 embryos were found to have emerged (Fig. 1A). The highest 25% embryos emerged in MS medium supplemented with zeatin 2 mg L^{-1} and 3% sucrose followed by 1/2 MS supplemented with BA 1 mg L^{-1} + NAA 0.1 mg L^{-1} + 3% sucrose. MS media compositions failed to show any embryo emergence when supplemented with BA and IAA. Finally, three hybrid plants were obtained from the rescued ovules only when cultured in MS media supplemented with zeatin 2.0 mg L^{-1} + 3% sucrose (Fig. 1B). The same media formulations were also treated for tetraploid cross. The effect of different concentration and combination of phytohormones of BA, NAA, IAA and zeatin was observed on direct hybrid plant regeneration from ovule

Table 1: Effect of medium composition on direct hybrid plant regeneration from ovule in the cross between *F. tataricum*×*F. esculentum* in diploid species

Growth regulators	Ovules rescued	Embryos emerged	Embryos emerged (%)	No. of hybrids
1/2 MS + 3% sucrose				
+BA 10 mg L^{-1}+ NAA 0.5 mg L^{-1}*	12	2	16.66	-
+BA 2 mg L^{-1} + NAA 2 mg L^{-1}	12	1	8.33	-
+BA 1 mg L^{-1} + NAA 0.1 mg L^{-1}	16	3	18.75	-
+ NAA 0.1 mg L^{-1}	10	1	10.00	-
MS + 3% sucrose				
+ BA 4 mg L^{-1}	10	0	0.00	-
+ BA 2 mg L^{-1}+IAA 0.2 mg L^{-1}	12	0	0.00	-
+ Zeatin 2 mg L^{-1}	16	4	25.00	3
+ Zeatin 2 mg L^{-1} +IAA 0.2 mg L^{-1}	12	1	8.33	-
Total	100	12	12.00	3

*: 2% sucrose

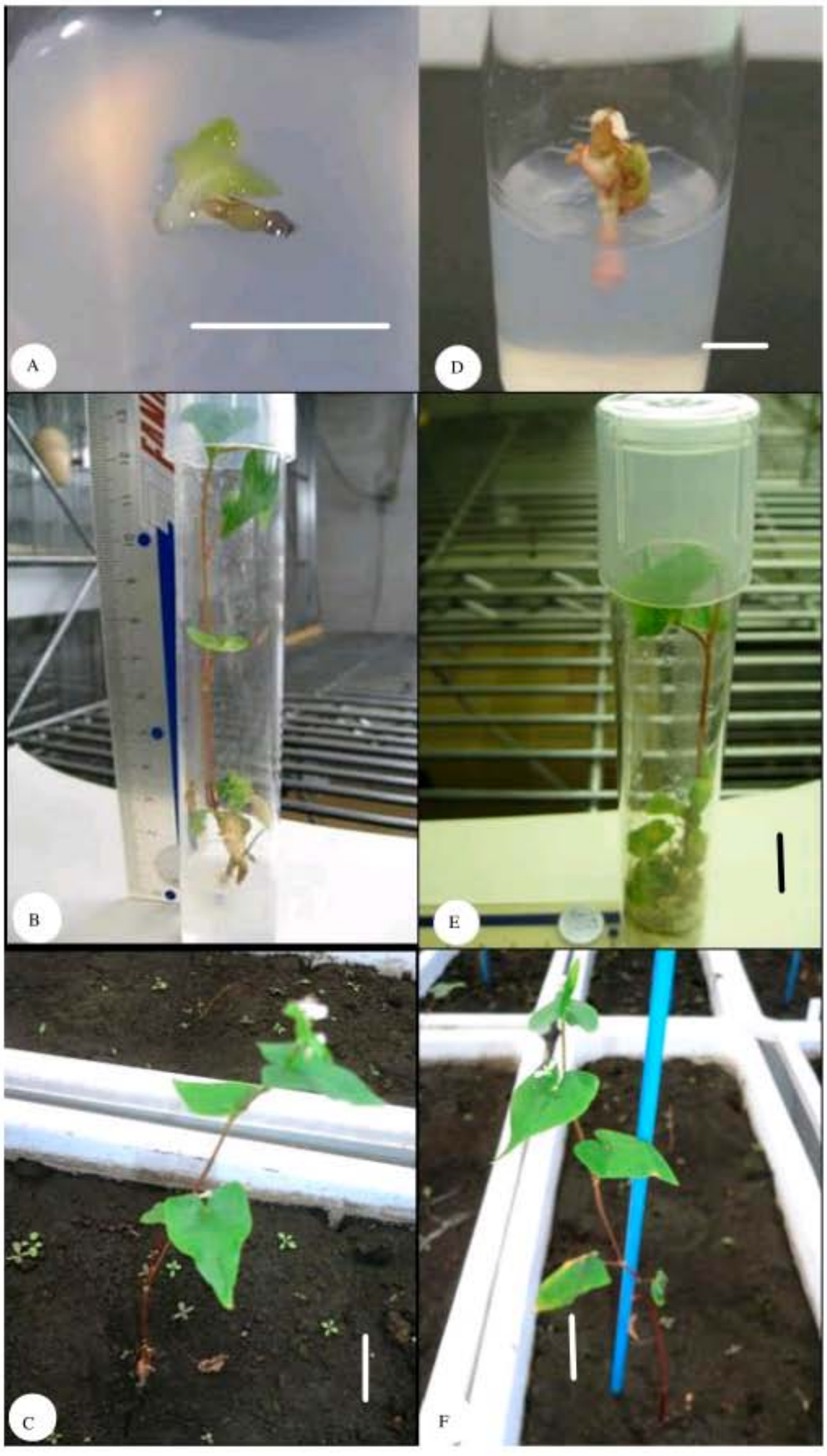

Fig. 1: Direct hybrid plant regeneration from ovule in the cross between *F. tataricum*×*F. esculentum* in diploid (A-C) and tetraploid (D-F). (A, D) Regeneration initiating stage, (B, E) Regenerated plantlet with root in the test tube. (C, F) Recovered hybrid grown in the greenhouse. The bars represent A and D = 1 cm and C, E and F = 2 cm

in the cross between tartary and common buckwheat in tetraploid species. It was observed that the composition of media is the most important factor in the success of interspecific cross from ovule culture of direct hybrid plant regenerations. Ovules were isolated and subjected to 1/2 MS and MS media supplemented with different concentrations of phytohormones and the results of the experiment are shown in Table 2. Eight combinations of the media were used in the cross between tartary and common buckwheat. 10 to 20 rescued ovules were treated for direct regeneration, but only 1-8 embryos were found to have emerged (Fig. 1D). The highest 40% embryos emerged in 1/2 MS supplemented with BA 1 mg L^{-1} + NAA 0.1 mg L^{-1} + 3% sucrose followed by 1/2 MS medium supplemented with BA 10 mg L^{-1} + NAA 0.5 mg L^{-1} and 2% sucrose. Therefore, 1/2 MS medium containing BA 1 mg L^{-1} + NAA 0.1 mg L^{-1} showed good results in embryos emerged and five hybrids were produced from the cultured ovules (Fig. 1E). In MS medium supplemented with zeatin 2 mg L^{-1}, two hybrids were recovered and the percentage of embryos emerged was 15%. This combination of both plody levels was very suitable for hybrid production from ovule. MS media compositions failed to show any embryo emergence when supplemented with BA and IAA. Finally, a total of seven hybrid plants were obtained from 100 ovules rescued. These were used with eight media combinations and the percentage of embryos emerged was 16%.

It was observed that hormone (NAA, BAP and zeatin) in the culture medium is a very important factor for the regeneration efficiency of direct hybrid plant regeneration from ovule culture. Investigation was made to determine suitable media formulation for the appropriate culture to accomplish successful regeneration of direct hybrid plant from ovule culture.

Interspecific hybridization between Tartary×Common buckwheat using indirect method: For callus induction, the ovules rescued from the cross of diploid species were treated with different concentrations and combinations of BA, NAA and 2, 4-D. These hormonal concentrations in the media, viz., 1/2 MS and MS were tested for their effect on the development of hybrid embryos and data were observed on rescued ovules, embryos emerged, texture of callus, color of callus and percentage of callus induction and the results are shown in Table 3. For callus induction, 7 to 12 rescued ovules were treated but found to get emerged only 1-4 embryos. Callus induction efficiency of ovules varied greatly in the culture media with different hormones. Callus was induced in three tested combinations but there was much variation in morphological nature and percentage of callus formation. For induction of callus from ovules, a combination of cytokinin (BA) and auxin (NAA) was more suitable than auxin alone in media composition. Highest (40%) callus formation was observed in medium containing 1/2 MS + BA 1 mg L^{-1} + NAA 0.2 mg L^{-1} + 3% sucrose followed by 1/2 MS + BA 0.1 mg L^{-1} + NAA 1 mg L^{-1} + 3% sucrose (Fig. 2A). Ten percent of callus formation was observed in 1/2 MS medium supplemented with a high concentration of BA 10 mg L^{-1} + NAA 0.5 mg L^{-1} + 2% sucrose. The texture of the callus was globular and compact and the color was greenish or reddish.

Table 2: Effect of medium composition on direct hybrid plant regeneration from ovule in the cross between *F. tataricum*×*F. esculentum* in tetraploid species

Growth regulators	Ovules rescued	Embryos emerged	Embryos emerged (%)	No. of hybrids
1/2 MS + 3% sucrose				
+BA 10 mg L^{-1}+ NAA 0.5 mg L^{-1}*	10	2	20	-
+BA 2 mg L^{-1} + NAA 2 mg L^{-1}	10	1	10	-
+BA 1 mg L^{-1} + NAA 0.1 mg L^{-1}	20	8	40	5
+NAA 0.1 mg L^{-1}	10	1	10	-
MS + 3% sucrose				
+ BA 4 mg L^{-1}	10	0	0	-
+ BA 2 mg L^{-1} + IAA 0.2 mg L^{-1}	10	0	0	-
+ Zeatin 2 mg L^{-1}	20	3	15	2
+ Zeatin 2 mg L^{-1} + IAA 0.2 mg L^{-1}	10	1	10	-
Total	100	16	16	7

*: 2% sucrose

Table 3: Effect of phytohormone on ovule rescue for callus induction in the cross *F. tataricum*×*F. esculentum* in diploid species

Growth regulators	Ovules rescued	Embryos emerged	Texture of callus	Color of the callus	Callus induction (%)
1/2 MS + 3% sucrose					
+BA 10 mg L^{-1} + NAA 0.5 mg L^{-1}*	10	1	Glo. and com.	Gre. and redi.	10
+BA 1 mg L^{-1} + NAA 0.2 mg L^{-1}	10	4	Globular	Gre. and redi.	40
+BA 0.1 mg L^{-1} + NAA 1 mg L^{-1}	12	2	Compact	Reddish	16.66
+2,4-D 1 mg L^{-1}	7	-	-	-	-
+2,4-D 1 mg L^{-1} +NAA 0.5 mg L^{-1}	7	-	-	-	-
MS + 3% sucrose					
+ 2,4-D 1 mg L^{-1}	10	-	-	-	-
Total	56	7	-	-	12.5

*: 2% sucrose, Glo: Globular, Com: Compact, Gre: Greenish, Redi: Reddish, Su: Sucrose

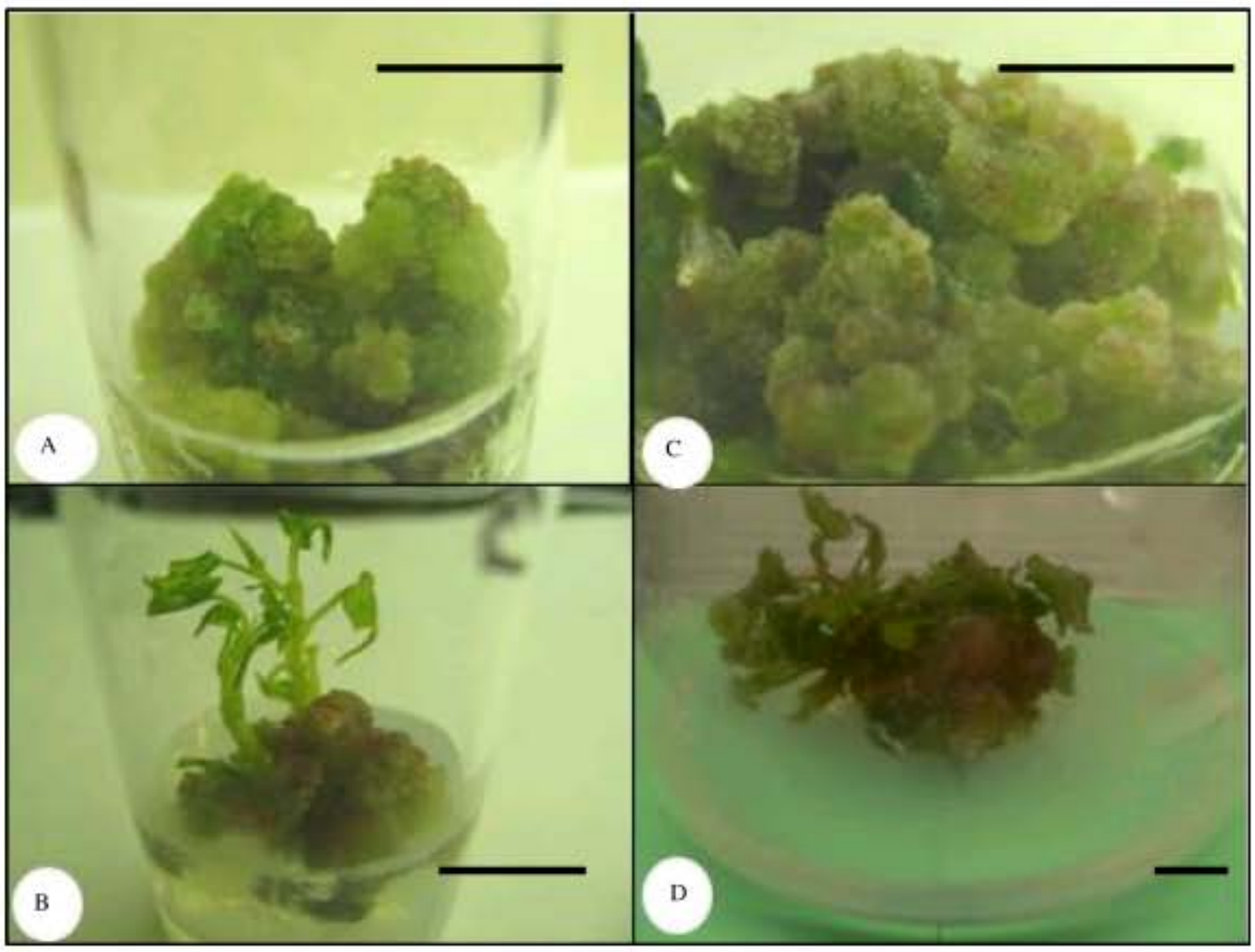

Fig. 2: Indirect plant regeneration through ovule culture in the cross *F. tataricum* × *F. esculentum* in diploid (A-B) and tetraploid (C-D). A, C: Globular greenish embryogenic callus. B, D: Embryo development and regeneration. The all bars represent 1 cm

Embryogenic response in the cultivation of embryos on 1/2 MS medium containing BA and NAA was observed. In this case, a globular, greenish type of callus was seen to be regenerable. This result is also supported by Samimy *et al*. (1996). A satisfactory amount of globular embryos was produced in 1/2 MS medium supplemented with BA 1 mg L^{-1} + NAA 0.2 mg L^{-1} + 3% sucrose (Fig. 2A). Globular structures were gradually appeared on the callus surface. 2,4-D was incorporated singly into both 1/2 MS and MS media in the concentration of 1 mg L^{-1} + 3% sucrose and did not evoke any response from the cultured ovules. Likewise, 2,4-D 1 mg L^{-1} + NAA 0.5 mg L^{-1} + 3% sucrose in the 1/2 MS medium failed to evoke any response. Tetraploid cross were also treated with the same media concentrations and combinations and the results are shown in Table 4. 1/2 MS +BA 1 mg L^{-1}+ NAA 0.2 mg L^{-1} + 3% sucrose was found to be most efficient for callus induction and the highest (50%) frequency of callus formation was recorded (Fig. 2C). Both plody level and concentration for this combination were very effective for callus induction from ovule in cross between tartary and common buckwheat. In 1/2 MS medium containing BA 10 mg L^{-1} + NAA 0.5 mg L^{-1} + 2% sucrose, the percentage of callus induction was 20% followed by BA 0.1 mg L^{-1}+NAA 1 mg L^{-1} + 3% sucrose. Only 2-5 embryos were found to have emerged from 10 to 20 rescued ovules treated for callus induction. In both crosses the texture and color of callus were similar. In 1/2 MS and MS media the growth regulators 2,4-D were presented in tetraploid cross and likewise, 1/2 MS with 2,4-D 1 mg L^{-1} + NAA 0.5 mg L^{-1} + 3% sucrose also failed to produce any response.

In this case for regeneration, a number of factors including genotype, developmental stage of the cell, nature of the explant and hormonal composition and concentration of the medium had been studied to improve the frequency of plant regeneration (Datta *et al*., 1990; Chowdhury *et al*., 1993). In the present study it was proved that use of zeatin and IAA in MS medium and BA and NAA in 1/2 MS medium induced shoot development from ovule-derived callus. Zeatin, IAA, BA and NAA were tested in the shoot-inducing medium to determine the optimum combination and concentration for shoot induction. Regeneration performance of ovule-derived callus and results are shown in Table 5. When the calli were transferred to MS medium supplemented with zeatin 2 mg L^{-1} + IAA 0.2 mg L^{-1} and within 4 weeks after subculture in the same medium, shoot proliferation started within two weeks of subculture. This result is also supported by Rumyantseva *et al*. (1995). The same procedure was also followed with tetraploid cross. The highest percentage of callus-produced plantlets/shoots (3.6) and the maximum number of shoots per culture (4.5)

Table 4: Effect of phytohormone on ovule rescue for callus induction in the cross *F. tataricum*×*F. esculentum* in tetraploid species

Growth regulators	Ovules rescued	Embryos emerged	Texture of callus	Color of the callus	Callus induction (%)
1/2 MS + 3% sucrose					
+BA 10 mg L^{-1} + NAA 0.5 mg L^{-1}*	10	2	Glo. and com.	Gre. and redi.	20
+BA 1 mg L^{-1} + NAA 0.2 mg L^{-1}	10	5	Globular	Gre. and redi.	50
+BA 0.1 mg L^{-1} + NAA 1 mg L^{-1}	18	2	Compact	Reddish	11.11
+2,4-D 1 mg L^{-1}	10	-	-	-	-
+2,4-D 1 mg L^{-1} +NAA 0.5 mg L^{-1}	10	-	-	-	-
MS+3% sucrose					
+ 2,4-D 1 mg L^{-1}	20	-	-	-	-
Total	78	9	-	-	11.54

*: 2% sucrose, Glo: Globular, Com: Compact, Gre: Greenish, Redi: Reddish

Table 5: Effect of phytohormone on ovule rescue for callus regeneration in the cross *F. tataricum*×*F. esculentum* in diploid species

Growth regulators	Shoot formation (%)	No. of shoot/callus	After culture No. of hybrids
MS + 3% sucrose			
+ Zeatin 2 mg L^{-1}	-	-	-
+ Zeatin 2 mg L^{-1}+IAA 0.2 mg L^{-1}	3.6	4.5	2
1/2 MS + 3% sucrose			
+BA 1 mg L^{-1} + NAA 0.1 mg L^{-1}	1.8	3.5	1
Total			3

*: Shoot formation (%), (No. of callus/No. of ovules rescued) × 100

Table 6: Effect of phytohormone on ovule rescue for callus regeneration in the cross *F. tataricum*×*F. esculentum* in tetraploid species

Growth regulators	Shoot formation (%)	No. of shoot/callus	After culture No. of hybrids
MS + 3% sucrose			
+ Zeatin 2 mg L^{-1}	-	-	-
+ Zeatin 2 mg L^{-1}+IAA 0.2 mg L^{-1}	5.1	4.5	2
1/2 MS + 3% sucrose			
+BA 1 mg L^{-1} + NAA 0.1 mg L^{-1}	3.8	3.5	2
Total			4

*: Shoot formation (%), (No. of callus/No. of ovules rescued)×100

were recorded in MS + zeatin 2 mg L^{-1} + IAA 0.2 mg L^{-1} media (Fig. 2B). Like callus induction, shoot regeneration was also found to have improved when 3% (w/v) of sucrose was used. Another treated medium combination-MS + zeatin 2 mg L^{-1} +3% sucrose-has totally failed to show any performance in regeneration. The morphology of calli also changed when subcultured on treated regeneration medium. Some calli proliferated and developed nodular structures and subsequently embryo-like structures in globular shapes, while some calli development stopped in compact shapes. This result is also supported by Samimy *et al*. (1996). Morphologically normal shoots were again subcultured in 1/2 MS medium without hormone. Finally, treated regeneration medium two and one hybrid plants were obtained from the ovule-derived callus when cultured in MS media supplemented with zeatin 2.0 mg L^{-1} +IAA 0.2 mg L^{-1} + 3% sucrose and 1/2 MS + BA 1 mg L^{-1} + NAA 0.1 mg L^{-1} + 3% sucrose, respectively. The best results for plant regeneration from ovule-derived callus were obtained in MS medium containing a combination of zeatin 2 mg L^{-1}+ IAA 0.2 mg L^{-1} + 3% sucrose (Table 6). In this treatment, the frequency of shoot regeneration was recorded as 5.1% and the maximum average number of shoots per callus was 4.5 (Fig. 2D). The plody levels for both Tartary and common buckwheat at this combination and concentration were very effective for regeneration. The percentage of shoot formation was 3.8% on 1/2 MS medium supplemented with BA 1 mg L^{-1} + NAA 0.1 mg L^{-1} + 3% sucrose and the per-callus maximum number of shoots was 3.5. MS + zeatin 2 mg L^{-1} +3% sucrose also totally failed to show any performance in regeneration. Tetraploid cross also showed morphological change in calli when the calli were transferred to treated regeneration medium. Four hybrid plants were recovered from tested regeneration media MS + zeatin 2.0 mg L^{-1}+ IAA 0.2 mg L^{-1} + 3% sucrose and 1/2 MS + BA 1 mg L^{-1}+ NAA 0.1 mg L^{-1} + 3% in both combinations.

Beasley and Ting (1973) and Beasly (1971) employed the ovule culture method for extensive studies on the effect of phytohormones on fiber and seed development in cotton. Beasley and Ting (1974) and Thengane *et al*. (1986) observed that unfertilized cotton ovules enlarged in the presence of cytokinin. According to them, cytokinin or auxin, singly or in combination, were used for callus induction and callus regeneration in cotton ovules. The success in plant ovule culture technology is greatly dependent on the choice of nutritional components and growth regulators in cultured media.

In the present investigation for two methods the media (1/2 MS and MS) showed positive responses when containing NAA, BA, IAA and zeatin. They were tested to evaluate their potentialities for direct regeneration, callus induction and callus regeneration from ovule culture. These two ovule culture methods are

very important for interspecific hybridization in *Fagopyrum tataricum*×*F. esculentum* in diploid and tetraploid species. For direct regeneration, callus induction and callus regeneration, cytokinin (BA) with auxin (NAA) and zeatin and IAA, produced uniformly good responses from the ovule culture. Therefore, the appropriate concentrations and combinations of hormone in media play an important role in regeneration from cultured ovule. A similar method was performed in an interspecific cross between *Lycopersicon esculentum* and *peruvianum*-complex species and hybrid plants were successfully produced (Egashira *et al*., 1999).

The results of the current investigations have shown that the 1/2 MS and MS media with appropriate concentrations and combinations of hormone make it possible to produce hybrid plants from rescued ovule of buckwheat.

In vitro ovule culture technique for producing interspecific hybrid between tartary buckwheat and common buckwheat has been established but no single medium was found adequate to ensure complete development of the fertilized ovules to plantlets. In rescued ovule, the direction of plant recovery determines how many steps of the media are required. It can be that one plant is obtained from one embryo or one embryo develops into more than one colonial plant through either callus regeneration or shoot multiplication. For instance, two steps of media were used in sunflower and wheat × rye crosses (Krauter *et al*., 1991). Three steps of media were used in wide cross of brassica (Gundimeda *et al*., 1992). In the present study, most of the hybrids produced for the crosses between tartary and common buckwheat (both diploid and tetraploid) were obtained by three steps of media: firstly, direct regenerating and rooting and secondly, callus inducing, callus regenerating and rooting. A similar result was also reported by Samimy *et al*. (1996).

As the development of the roots was poor on the culture medium, the elongated shoots were transferred to rooting medium for root induction. Treatment for root induction was obtained in ½ MS + NAA 0.1 mg L^{-1} + 2% sucrose (Fig. 1B, E, 3A, C). Full root development was

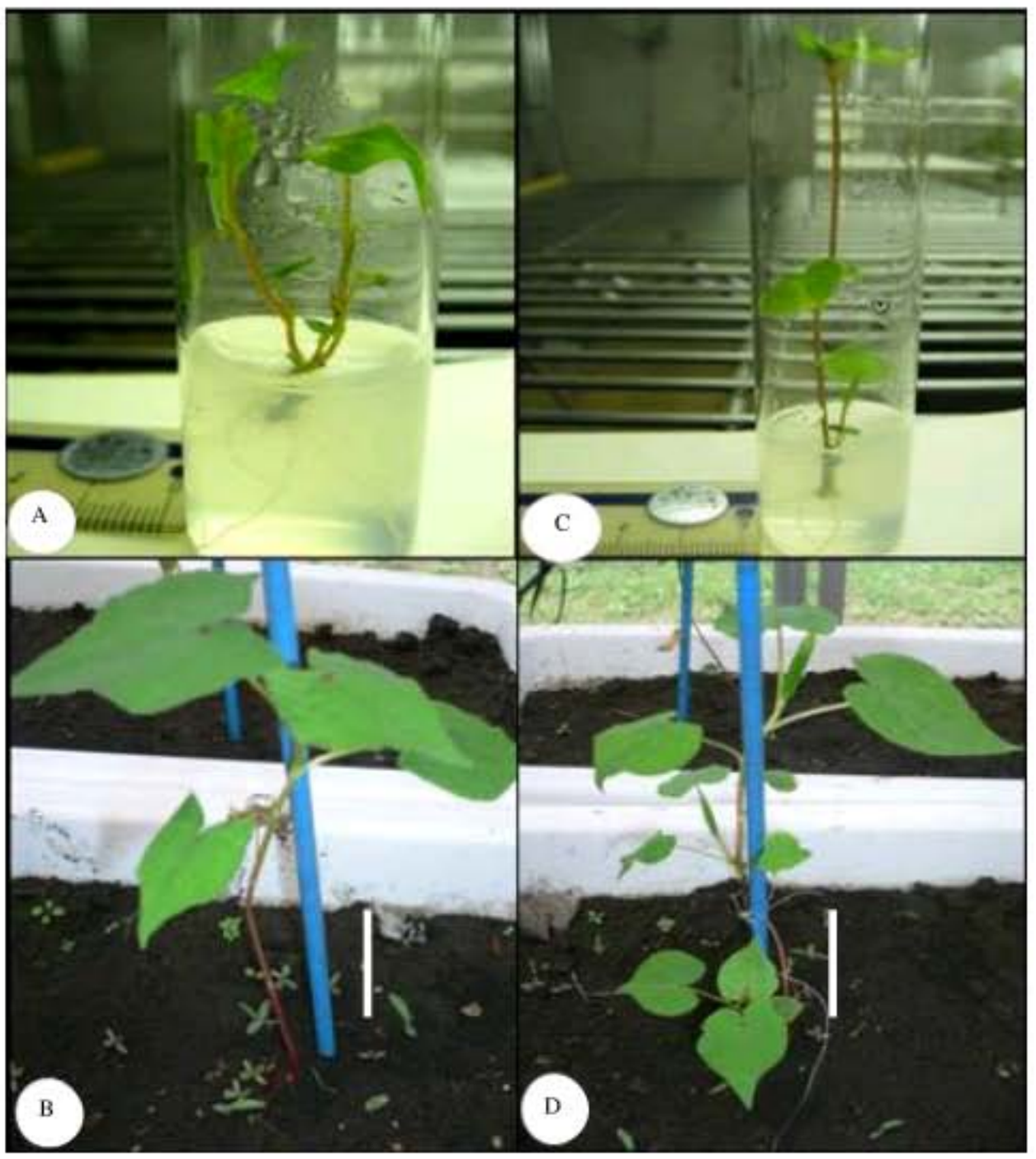

Fig. 3: Regenerated plant through ovule culture in the cross *F. tataricum*×*F. esculentum* in diploid (A-B) and tetraploid (C-D). (A, C) Regenerated plantlet with root in the test tube. (B, D) Recovered hybrid grown in the greenhouse. The bars represent B, D = 2 cm

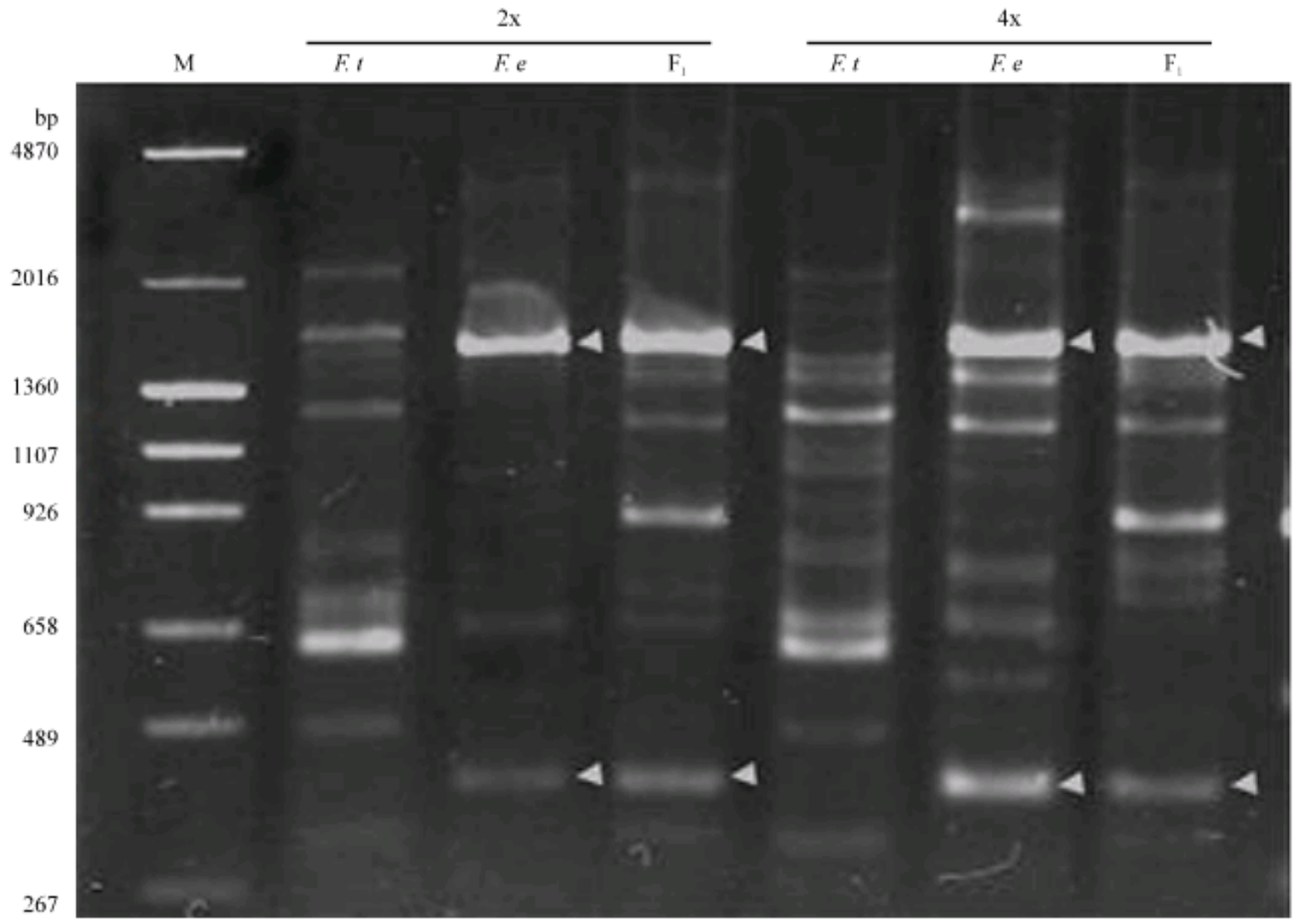

Fig. 4: Polymorphism of interspecific hybrids between *F. tataricum* and *F. esculentum* both diploid and tetraploid based on RAPD primer OPK15 (CTCCTGCCAA). M: Molecular weight marker, *F. t*: *F. tataricum*, *F. e*: *F. esculentum*

observed within two week of subculture. *In vitro* rooted plantlets were acclimatized carefully and planted in plastic pots containing sterilized vermiculite. The vermiculite method produced root development and plantlets that survived well and two weeks later the rooted plants with well-developed shoots were transferred to soil in the greenhouse (Fig. 1C, F, 3B, D).

Finally, confirming hybridity of the regenerated plant was identified through DNA banding patterns resulting from RAPD-PCR analysis of the parent. In the RAPD-PCR analysis, all the DNA bands amplified from the male parent (*F. esculentum*) were also clearly amplified in the interspecific F_1 hybrids (Fig. 4). Primer that amplified bands specific to the male parent might reveal a proper pattern of a true hybrid. These results confirmed that rescued ovule plants from the cross tartary and common buckwheat of both plody levels were true interspecific F_1 hybrid plants. For testing hybrid purity in buckwheat, RAPD markers were effectively used by Wang *et al*. (2004).

In the present study, ovule culture method to produce interspecific hybrid between *F. tataricum*× *F. esculentum* was established. This results in the first success in the cross between these two species and may bring a major breakthrough in buckwheat breeding. This study suggested that the success of interspecific hybridization in *Fagopyrum tataricum*× *F. esculentum* in diploid and tetraploid species through ovule culture technology is greatly dependent on the choice of nutritional components and growth regulators in cultured media. In the present investigation, the appropriate concentrations and combinations of hormone in media play an important role for plants regeneration from ovule culture. Therefore, the two ovule culture methods are very important, because no single medium was found adequate to ensure complete development of the fertilized ovules to plantlets.

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