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Effects of 2, 4-D and 6-BAP on callus induction of *Fagopyrum esculentum* (Buckwheat): An embryo culture technique for regeneration and conservation

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ABSTRACT
In the present study, we assessed the effects of 2, 4-D and 6-BAP concentration in immature embryo culture technique for callus regeneration. The hormonal concentrations i.e., 2, 4-D (0.5 mg/L, 1.0 mg/L & 2.0 mg/L) and 6-BAP (1.0 mg/L, 2.0 mg/L & 3.0 mg/L) with their interaction combination were tested. Notably, the 1 mg/L 2, 4-D and 6-BAP 3 mg/L were found significantly suitable for enhancing the callus formation percent with 91.67% and 100%, respectively ($p<0.05$). Similarly, hormonal interaction effect of 1 mg/L 2, 4-D with 2 mg/L 6-BAP was found suitable for callus regeneration (98.33%) and embryoids formation ($p<0.05$) as compared to control (MS medium without hormone supplement). The implementation of these results must be suggested for conservational management aspects, especially callus and embryoids formation through immature embryo rescue culture and development of interspecific compatible hybrid morph for potential donors to transfer its desirable traits of high productivity, self-pollination ability, frost resistance, insects attack, overall plant vigor and ultimately higher quality yield. The cross-incompatibility barriers may overcome through embryo rescue technique.
Keywords: *Embryo Culture, 2, 4-D, 6-BAP, Embryo abortion, Embryoids, Callus regeneration.*

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INTRODUCTION

Common buckwheat or ogal belongs to Polygonaceae. It is main common traditional crop species used for food and cultivated in Southeast Asia [1] as playing a pivotal role in sustaining the livelihood of poor marginal farmers in most of the food deficit areas in many of the higher altitudinal mountainous areas along with Indian Himalayan Regions (IHR). Buckwheat is categorized as a pseudo cereal because its seed structurally and chemically resembles with the cereals [2]. The seeds contain 70–91% starch [2] amylose and amylopectin [3], high concentration of all essential amino acids [4], minerals [4], the herb riches in antioxidant [5] *and aromatic compounds [6].*

Notably, the reproductive biology of the buckwheat is cross-pollinating due to heterostylous nature of flowers, incompatible pollination and fertilization [7] however, the superior genotypes of buckwheat can't be easily maintained and propagated due to heterostylic self and cross-incompatibility nature [8], so, it is considered a neglected crop and has never attained the status of major cereal crop from the production and yield point of view. Therefore, the inter-specific crosses with wild buckwheat species may be potential donors to transfer its desirable traits [9, 10] but, the hybridization of buckwheat cultivars with wild species is limited due to the cross-incompatibility barriers [11]. However, the introduction of desirable agronomic traits from the wild species into the cultivated varieties may be attained by embryo rescue technique for developing compatible morphs has been studied on buckwheat by many researchers [12-14, 15] but there are no reliable and efficient protocol about selection of 2, 4–D and 6-BAP hormone with concentration on embryo rescue culture for regeneration of callus or embryoids. Therefore, the present research has been made to analyse the effects of 2, 4-D and 6-BAP and standardized their concentration in immature embryo culture technique for callus regeneration.

MATERIALS AND METHODS

Procurement, Sterilization and Inoculation of Planting Material

All instruments were autoclaved for complete sterilization. Immature seeds were harvested and disinfected for 10 min in 2% NaOCl solution followed by sterilized with 70% ethanol for 5 min then the pericarp was removed, the peeled seeds were further sterilized for 5 min in 1% NaOCl solution and washed three times with sterile distilled water, respectively. Next, the immature embryos aseptically rescued followed by surface sterilized in 70% Ethanol for 1 min followed by rinsed five times in sterile distilled water. Sterilized immature embryos were cultured on Murashige & Skoog medium [16] (with 0.8% agar and 3% sucrose) supplemented with different concentration of 2,4-D (0.5 mg/L, 1.0 mg/L and 2.0 mg/L) and 6-BAP (1.0 mg/L, 2.0 mg/L and 3.0 mg/L) along with combination and control (MS medium without hormone supplement). The culture pH was adjusted to 5.8 before autoclaving (121 ^{o}C for 20 min at 15 psi). The experiments were performed in conical flask (100 ml volume, 25 ml medium), consist of twelve embryos/treatment (4 embryos/flask and repeated thrice) with three replicates using complete randomized design (CRD) in factorial concept for analyzed the data statistically. The isolated embryos were cultured in growth chamber at 25 ± 2 °C in complete dark condition. According to the treatment the immature embryos had formed callus or embryoids to the 5-10 mm sized were considered as embryoids formation [11]. The mean daily callus formation rate was calculated using following formula:

$$MDC = \frac{CFP}{D}$$

Where, MDC mean daily callus formation rate; CFP is callus formation percentage and D is days required for complete regeneration.

Statistical Analysis

Data were processed by the analysis of variance (ANOVA) on the basis of completely randomized design (CRD) with 3 replications. The data were analyzed using computer SPSS software (version 20), and the means were compared by Duncan's multiple range test ($P < 0.05$ level).

RESULT AND DISCUSSION

The immature embryos on MS medium supplemented with 2, 4-D 1.0 mg/L showed maximum (91.67%) callus formation with maximum mean daily callus formation (MDC) rate (7.38) with minimum inoculation period (5.33 days) ($p<0.05$) followed by 0.5 mg/L (75.00%) and 2.0 mg/L (66.67%) Analysis of variance showed no significant effects on days required for onset of germination but significant to the other parameters ($p<0.01$) (Tab 1). Whereas, 3.0 mg/L 6-BAP showed maximum (100%) embryoids formation with high MDC rate (9.39) significantly ($p<0.05$) followed by 2.0 mg/L (83.33%) and 1.0 mg/L (75.00%). Similarly, ANOVA showed significant effects ($p<0.01$) between and within the groups in all observed parameters as compared to control (Tab 2). The minimum inoculation period was found in 2.0 mg/L 6-BAP (9.00 days) significantly ($p<0.05$) rather than other treatments (see Tab. 2). Notably, the interaction effects of 1.0 mg/L 2, 4-D with 2.0 mg/L 6-BAP showed best callus formation (98.33%) with high MDC rate (9.23) with minimum inoculation period (8.33 days) significantly ($p<0.05$) followed by 2, 4-D 1.0 mg/L with 6-BAP 3.0 mg/L and 2, 4-D 2.0 mg/L with 6-BAP 1.0 mg/L (83.33% in both treatment). ANOVA showed significant effects between groups and within groups for all interaction combination of both hormone ($p<0.01$) as compared to control [see Tab. 3 & Fig. A (b)].

Table 1. Response of immature embryos on MS Medium supplemented with various concentration of 2, 4-D.

2, 4-D (mg/l)	Days required for onset of regeneration (d)	Days required for complete regeneration (d)	Inoculation period (d)	Callus Induction (%)	MDC
MS	11.00±1.00b	27.67±2.52c	16.67±3.06c	2.33±0.58a	0.09±0.03a
0.5	9.33±2.31ab	21.33±2.31b	12.00±0.00b	75.00±0.00bc	3.54±0.36b
1.0	7.67±4.16ab	13.00±2.65a	5.33±1.53a	91.67±14.43c	7.38±2.51c
2.0	4.67±1.53a	11.33±0.58a	6.67±2.08a	66.67±14.43b	5.87±1.18bc
f-value	3.37	36.54	20.31	44.06	15.48
p-value	0.075^{ns}	0.000**	0.000**	0.000**	0.000**

Symbols used: 2, 4-D = 2, 4-Dichlorophenoxyacetic acid; MS = Murashige and Skoog medium (control without hormone supplement); MDC = mean daily callus formation.

Note: same letter (s) in the same column indicate (s) insignificant differences and data are shown in mean ± SD at $p<0.05$ level using Duncan's multiple range test (DMRT); ** & ns means $p<0.01$ level of significance and non significance, respectively.

Table 2. Response of immature embryos on MS Medium supplemented with various concentration of 6-BAP.

6-BAP (mg/l)	Days required for onset of regeneration (d)	Days required for complete regeneration (d)	Inoculation period (d)	Callus Induction (%)	MDC
MS	9.33±0.58c	28.33±2.89c	19.00±2.65b	2.33±0.58a	0.08±0.03a
1.0	3.33±0.58b	21.33±2.31b	18.00±2.65b	75.00±0.00b	3.54±0.36b
2.0	2.00±1.00a	11.00±2.65a	9.00±2.00a	83.33±14.43b	7.66±0.61c
3.0	1.33±0.58a	10.67±0.58a	9.33±0.58a	100.00±0.00c	9.39±0.52d
f-value	80.00	42.01	19.13	107.12	269.54
p-value	0.000**	0.000**	0.001**	0.000**	0.000**

Symbols used: 6-BAP = 6-benzyl amino purine; MS = Murashige and Skoog medium (control without hormone supplement); MDC = mean daily callus formation.

Note: same letter (s) in the same column indicate (s) insignificant differences and data are shown in mean ± SD at $p<0.05$ level using Duncan's multiple range test (DMRT); ** means $p<0.01$ level of significance.

Table 3. Interaction effects (2, 4-D and 6-BAP) on various parameters of immature embryos of *F. esculentum* during embryo culture.

2, 4-D + 6-BAP (mg/l)	Days required for onset of regeneration (d)	Days required for complete regeneration (d)	Inoculation period (d)	Callus Induction (%)	MDC
MS	9.00±1.00c	28.33±2.89d	19.33±3.06d	2.33±0.58a	0.08±0.02a
0.5+1.0	3.33±0.58ab	21.33±2.31c	18.00±2.65d	62.33±2.52bc	2.95±0.40b
0.5+2.0	2.00±1.00a	11.00±2.65a	9.00±2.00ab	64.67±0.58c	6.10±1.37a
0.5+3.0	2.00±0.00a	11.33±0.58a	9.33±0.58ab	58.33±2.89b	5.15±0.26c
1.0+1.0	3.00±1.00ab	15.33±0.58b	12.33±1.53bc	65.33±0.58c	4.26±0.12c
1.0+2.0	2.33±0.58ab	10.67±0.58a	8.33±1.15a	98.33±2.89e	9.23±0.24f
1.0+3.0	3.67±0.58b	16.33±1.53b	12.67±2.08bc	83.33±3.06d	5.13±0.55c
2.0+1.0	3.67±0.58b	17.00±2.00b	13.33±2.52c	83.33±3.06d	4.94±0.47cd
2.0+2.0	3.00±1.00ab	15.00±1.00b	12.00±1.73abc	87.67±2.52d	5.86±0.37d
2.0+3.0	3.00±1.00ab	14.67±0.58b	11.67±0.58abc	83.33±2.89d	5.68±0.03d
f-value	19.44	29.55	10.28	375.58	57.98
p-value	0.000**	0.000**	0.000**	0.000**	0.000**

Symbols used: 2, 4-D = 2, 4-Dichlorophenoxyacetic acid; 6-BAP = 6-benzyl amino purine; MS = Murashige and Skoog medium (control without hormone supplement); MDC = mean daily callus formation.

Note: same letter (s) in the same column indicate (s) insignificant differences and data are shown in mean ± SD at $p<0.05$ level using Duncan's multiple range test (DMRT); ** means $p<0.01$ level of significance.

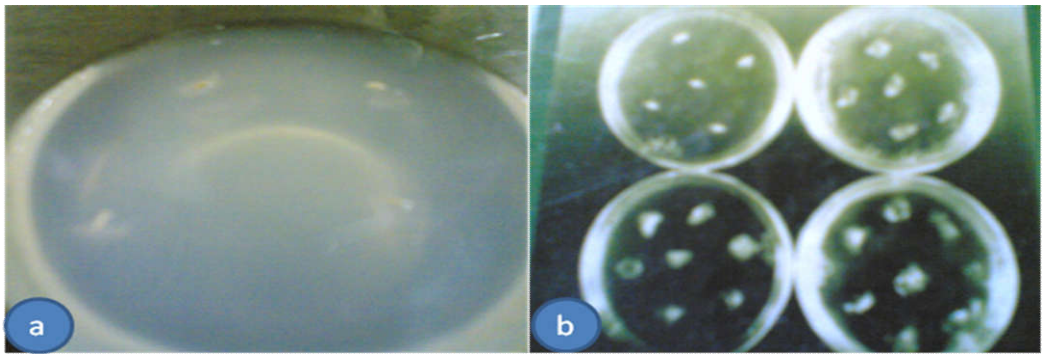

Fig 1: Embryo culture of *F. esculentum* (Buckwheat).

a. Response of immature embryos on MS medium (without hormone supplement) showing no callus induction.

b. Response of immature embryos on MS medium supplemented with 2, 4-D 1.0 mg/L and 6-BAP 2.0 mg/L concentration showing callus regeneration.

A high frequency of plant regeneration is one of the essential prerequisite for the application of tissue culture in crop improvement that is embryo culture. Previously, Woo [17] observed similar results with using 2, 4-D on white medium, similarly, Niroula [15] also showed maximum embryoids formation in 0.2 mg/L IAA with 2 mg/L 6-BAP supplemented with 3% sucrose on MS medium during embryo culture in *F. esculentum*. Similarly, 2, 4-D, IAA, IBA, NAA has been used as hormonal supplement on MS media in embryo culture along with 6-BAP for embryoids formation and found significant results [11, 12].

CONCLUSION

On the basis of the study it can be concluded that immature embryo culture in *F. esculentum* greatly influenced by 2, 4-D and 6-BAP. 1 mg/L 2, 4-D and 6-BAP 3 mg/L were found suitable for callus formation percent with 91.67% and 100%, respectively. The interaction effect of 1 mg/L 2, 4-D and 2 mg/L 6-BAP was found suitable for callus regeneration (98.33%). Use of hormonal combination of auxin (2, 4-D) and kinetin (6-BAP) was suggested for well development and regeneration of callus or embryoids production in *F. esculentum*.

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