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Biotechnological optimization of *In Vitro* embryo rescue in citrus: Impact of explant size and cytokinin gradients on germination efficiency

Shivam and Amarjeet Kaur*

PG Department of Agriculture (Horticulture), Khalsa College, Amritsar 143002, Punjab, India

ABSTRACT

This study investigated the biochemical impact of 6-benzylaminopurine (BAP) and initial explant size on the *in vitro* morphogenesis of five *Citrus* species: *C. aurantifolia*, *C. limon*, *C. sinensis* cv. Mosambi, *C. reticulata* cv. Kinnow, and *C. reticulata* Blanco cv. Nagpur mandarin. Mature embryos were cultured on Murashige and Skoog (MS) basal medium integrated with varying cytokinin gradients (0–3 mg/L BAP). Our data reveals a significant species-specific threshold for germination, with *C. limon* and *C. reticulata* cv. Kinnow achieving 100% efficiency at an optimal concentration of 2 mg/L BAP. Conversely, concentrations exceeding this limit (3 mg/L) induced an inhibitory effect, likely due to supra-optimal hormonal signaling. Furthermore, a positive correlation was established between explant dimensions and regenerative capacity; larger embryos (7.0–11.0 mm) consistently outperformed smaller cohorts (<5 mm), with *C. limon* reaching a peak germination rate of 98.46%. These findings provide a refined biotechnological protocol for embryo rescue, essential for overcoming reproductive barriers and accelerating genetic improvement programs in polyembryonic *Citrus* cultivars.

Key words: Embryo rescue, 6-benzylaminopurine (BAP), *in vitro* morphogenesis, *Citrus* species, explant physiology.

INTRODUCTION

Citrus remains a globally preeminent fruit crop, valued for its complex secondary metabolites and industrial utility. However, conventional breeding of many *Citrus* species is significantly impeded by nucellar polyembryony, a condition where multiple asexual embryos develop from the nucellus, often outcompeting and suppressing the weaker zygotic (hybrid) embryo. This competition for endosperm-derived nutrients limits the recovery of novel genotypes, creating a bottleneck for genetic diversity. To bypass these biological constraints, embryo culture a pivotal biotechnological tool is employed to excise and germinate embryos under aseptic *in vitro* conditions (Viloria *et al.,* 14). This technique is vital for "embryo rescue," allowing researchers to salvage hybrid plantlets that would otherwise abort due to incompatibility or dormancy. The success of this morphogenetic process is governed by two primary factors: the endogenous hormonal balance within the explant and the exogenous composition of the culture medium. Among synthetic growth regulators, the cytokinin 6-benzylaminopurine (BAP) plays a critical role in regulating cell division and apical dominance during *in vitro* development (Zhu *et al.,* 16). While Murashige and Skoog (MS) medium is a standard basal formulation for *Citrus* tissue culture, the specific requirement for BAP is often cultivar-dependent. Additionally, the physiological age and physical size of the embryo serve as indicators of its nutrient reserves and biochemical maturity; larger embryos typically exhibit higher morphogenetic potential due to a more robust endogenous phytohormone profile. Embryo culture, a vital technique within plant tissue culture, has been extensively employed to overcome barriers in hybridization and propagation (Dutt *et al.,* 4). It refers to one of the applications of plant tissue culture widely used with the purpose to shorten breeding cycle by overcoming dormancy and to culture in nucellar or zygotic embryo (Zulkarnain *et al.,* 17). It involves excising immature or mature embryos from seeds and growing them under aseptic in vitro conditions to produce viable plants and to obtain diploid hybrids from crosses between polyembryonic cultivars (Tan *et al.,* 12). This approach allows bypassing constraints such as embryo abortion and incompatibility barriers, thereby accelerating breeding cycles and ensuring recovery of hybrids that might otherwise fail to germinate naturally (Khan *et al.,* 7).

The success of in vitro embryo culture is influenced by several factors, including the physiological age and size of the embryo, species-specific responses, and the composition of the culture medium (Bond, 2; Carvalho *et al.,* 3). Among these, embryo size is considered a critical determinant of regeneration success, as larger embryos generally possess greater nutrient reserves and a more favorable endogenous hormonal balance, resulting in superior germination capacity and morphogenetic potential compared with smaller embryos (Kishore *et al.,* 8). In citrus,

*Corresponding author email: dr.amarjitkaur30@gmail.com

classification of embryos into different size categories has been useful for evaluating regeneration efficiency and identifying developmental stages suitable for successful establishment (Verma *et al.,* 13). Likewise, studies on embryo developmental stages combined with optimization of culture conditions in polyembryonic diploid citrus demonstrated that early cotyledonary embryos exhibited improved germination performance on Murashige and Skoog (MS) medium (Zhu *et al.,* 16).

Besides embryo developmental status, culture medium composition and plant growth regulators play an essential role in determining embryo germination and seedling development. Among the cytokinins, 6-benzylaminopurine (BAP) has shown significant effects in promoting embryo germination, enhancing shoot proliferation, and improving overall seedling vigor in citrus species (Liu *et al.,* 9). Murashige and Skoog medium (MS) (Murashige and Skoog, 10) remains the most widely used basal medium for culturing conserved citrus embryos and has proven effective in supporting embryo germination across diverse citrus genotypes. Although previous studies have independently demonstrated the importance of embryo developmental stage, embryo size, and cytokinin supplementation, there remains a limited understanding of their combined influence on embryo rescue efficiency across diverse Citrus taxa. In particular, systematic evaluation of how varying embryo sizes respond to different BAP concentration gradients for maximizing germination and regeneration efficiency is still insufficiently explored. Therefore, the present study aims to optimize a high-efficiency in vitro embryo rescue protocol by investigating the interactive effects of embryo size and BAP concentrations on embryo germination and seedling establishment in citrus. By identifying the optimal developmental stage and hormonal environment required for successful embryo recovery, this study seeks to facilitate triploid breeding programs and accelerate the propagation of elite seedless citrus cultivars.

MATERIALS AND METHODS

For an embryo culture, the fruits were collected and surface-sterilized by washing with water and detergent (Teepol-20 ml) and then surface sterilized with 0.1% $HgCl_2$ for 8-10 minutes. Finally, the fruits were rinsed three times with sterile water, under aseptic conditions. The fruits were cut, in sterile conditions, at the equatorial zone, avoiding the core where seeds are embedded, and opened from which the immature embryos were excised carefully, under a stereomicroscope, from the micropylar end of the seed, after removing the seed coat. From the excised embryos the embryos size was measured with the help of graph paper.

The inoculation media contained MS (Murashige and Skoog, 10) medium alone and also MS medium supplemented with various 6-benzylaminopurine (BAP) concentrations. After that the adjustment of pH (Century CP931, Chandigarh) to 5.7 with 1 N NaOH (sodium hydroxide), the media were sterilized in an autoclave, at 121˚C for 30 minutes at 1.06 kg/cm^2 (15 PSI) pressure, and were dispensed in test tubes. Laminar air flow cabinet (Klenzaids, Bombay) was used to culture embryos of various citrus species. Embryos were grown at 25 ± 1˚C with white light (5,000 lux) and a 16-hours photoperiod**.**

One embryo per test tube were cultured for germination and plant growth. The effect of embryo size and culture medium on germination percentage and on plant growth after culturing for 4 weeks in growth medium. To study the optimum developmental stage, we used 10 tubes per treatment. The effects of embryo developmental stage on the survival, germination and later embryo growth were recorded. The experimental data were subjected to statistical analysis following a Completely Randomized Design (CRD) to assess the significance of treatment effects. Mean values of regenerated shoots, leaves and roots along with their corresponding standard errors (SE) were computed to evaluate the variability and reliability of the results. For multiple mean comparisons, Duncan's Multiple Range Test (DMRT) was employed using the 'Agricolae' package in R Studio, providing a robust method for identifying statistically significant differences among treatment groups at the specified confidence level.

RESULTS AND DISCUSSION

Embryo culture proved highly effective in enhancing germination across all citrus species tested. The treatment MS + 2.0 mg/L BAP showed a statistically significant increase in germination percentage ($P < 0.05$) across all species compared to the control. The supplementation of MS medium with BAP significantly improved germination percentages, with 2 mg/L BAP identified as the optimal concentration. Beyond this level, germination declined in all cultivars, indicating a suppressive effect of higher BAP concentrations. These observations agree with earlier findings (Verma *et al.,* 13) but contrast with Liu *et al.* (9), who reported enhanced embryo germination at higher BAP levels (4.0 mg/L). Such contradictions emphasize that the response to growth regulators is species- and cultivar-specific (Gercheva *et al.,* 5).

Among the cultivars, *Citrus limon* and *C. reticulata* cv. Kinnow divulged the highest germination (100%)

at 2 mg/L BAP, while *C. reticulata* Blanco cv. Nagpur mandarin showed the lowest response (51.85%) (Table 1). The decline in germination at higher BAP concentrations (3.0 mg/L) may be attributed to the supra-optimal levels of cytokinins, which can sometimes lead to hormonal imbalance or 'vitrification' (glassiness) of the embryonic tissues, a phenomenon also observed by Pérez-Tornero and Porras (11) in lemon culture. The overall results clearly establish that the embryo culture consistently outperformed untreated controls, where germination percentages were markedly lower in the absence of BAP which is also proven by statistical analysis.

Embryo size exerted a strong influence on germination success in various citrus species (Table-2). Larger embryos (7.0–11.0 mm) demonstrated the highest germination rates, reaching up to 98.46% in *C. limon*, (Fig.1) while smaller embryos (<5 mm) germinated at much lower frequencies (Table 2). This positive correlation between embryo size and germination agrees with previous reports (Carvalho *et al.,* 3; Blanchard et al*.,* 1; Kishore *et al.,* 8). The superior response of large embryos may be attributed to their greater endogenous hormonal balance and nutrient reserves that promote morphogenesis. In contrast, smaller embryos likely faced developmental constraints, leading to reduced germination.

The present findings highlight that embryo culture, particularly using larger embryos on MS + 2 mg/L BAP, ensures maximum and uniform germination in citrus species. Variability among cultivars indicates underlying genotypic differences (Wakana *et al.,* 15; Ghorbel *et al.,* 6), reinforcing the need for cultivar-specific optimization of culture conditions.

After 8-10 weeks of culturing when a considerable amount of shoots and roots were developed the plantlets were removed from vial with special care without any damage to the formed roots. After removal the roots were washed with running tap water to remove the media from plantlets. In the meantime, the plantlets were transferred to disposable glasses filled with sterilized soil: cocopeat

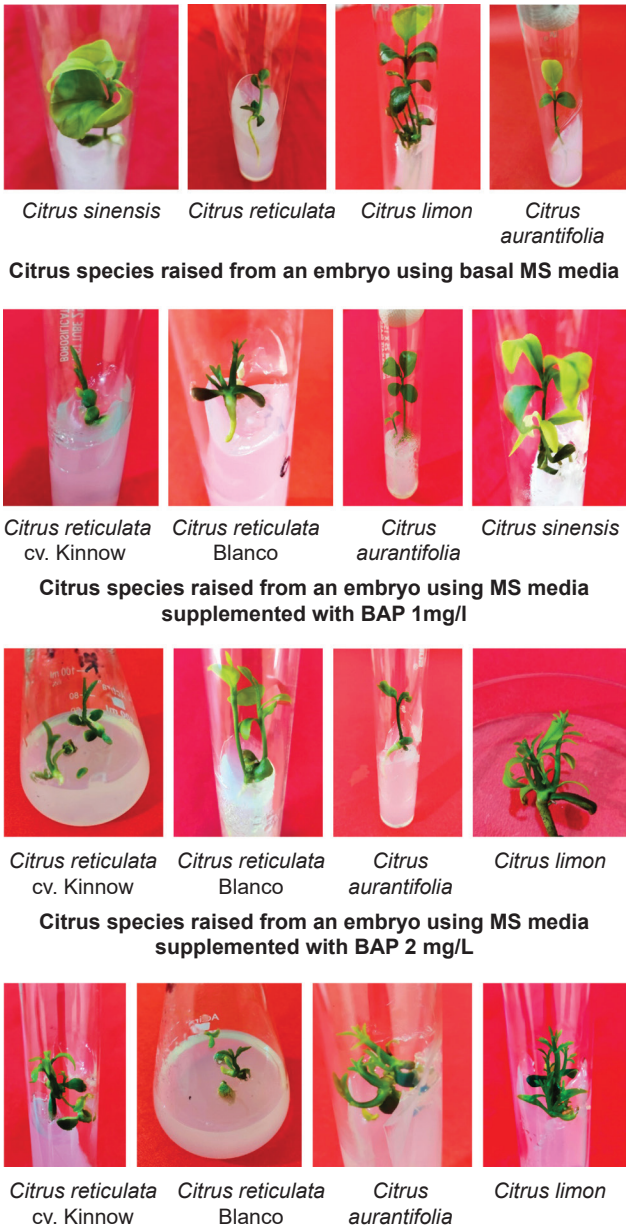

Fig. 1. Citrus species raised from an embryo using MS media supplemented with BAP 3mg/L.

Table 1: Comparative germination efficiency across *Citrus* species under varying BAP gradients.

S. No.	Citrus species	No. of embryos cultured	Germination (%)			
			MS+ BAP			
			0 mg/L	1 mg/L	2 mg/L	3 mg/L
1.	*Citrus aurantifolia*	15	75.67	86.66	93.32	79.00
2.	*Citrus limon*	15	90.68	100.00	100.00	93.33
3.	*Citrus sinensis* cv. Mosambi	15	60.96	70.00	73.34	61.53
4.	*Citrus reticulata* cv. Kinnow	15	90.78	100.00	100.00	92.00
5.	*Citrus reticulata* Blanco cv. Nagpur mandarin	15	41.62	56.25	60.00	51.85

Table 2: Influence of initial explant size on *In Vitro* morphogenetic response.

S. No.	Citrus Species	Embryo size (mm)	No. of embryos inoculated /treatment	No. of embryos germinated (MS+BAP)				Germination (%)			
				0 mg/l	1 mg/l	2 mg/l	3mg/l	0 mg/l	1 mg/l	2 mg/l	3 mg/l
1.	*Citrus aurantifolia*	2.0 - 5.0	30	9	12	19	11	30.00	40.00	63.33	36.67
		5.1 - 7.0	20	11	14	15	12	55.00	70.00	75.00	60.00
		7.1-9.0	25	17	20	23	18	68.00	80.00	92.00	72.00
2.	*Citrus limon*	3.0-5.0	60	24	31	34	28	40.00	51.60	56.67	46.66
		5.1-8.0	80	55	60	65	57	68.75	75.00	81.25	71.25
		8.1-11.0	65	51	59	64	54	78.46	90.76	98.46	83.07
3.	*Citrus sinensis* cv. Mosambi	3.0-5.0	20	4	7	9	5	20.00	35.00	45.00	25.00
		5.1-8.0	18	7	12	15	9	38.88	66.67	83.34	50.00
		8.1-11.0	19	9	16	18	12	47.37	84.21	94.70	63.16
4.	*Citrus reticulata* cv. Kinnow	3.0-6.0	50	10	15	19	11	18.00	30.70	38.00	22.00
		6.1-7.0	60	24	31	35	27	40.00	51.67	58.33	45.00
		7.1-9.0	50	32	39	43	35	64.00	78.00	86.00	70.0
				21.50 ± 5.12^{d}	27.60 ± 4.85^{b}	32.70 ± 5.47^{a}	24.90 ± 4.93^{c}	48.19 ± 4.85^{d}	59.47 ± 4.12^{b}	69.78 ± 5.24^{a}	54.98 ± 4.67^{c}

Note: Combined Mean Analysis indicates a highly significant (P<0.001) peak at 2.0 mg/L BAP (69.78 ± 5.24a) compared to the control (48.19 ± 4.85d); 2.0 mg/L BAP (5.47) also gave higher number of embryo germinated.

(1:1) which ultimately were transferred to shade house for acclimatization. In the shade house, the top of the pots was covered with transparent plastic sheet and grew at room temperature for 14 days with periodic irrigation (2 days interval). The highest survival percentage was reported in *Citrus limon* i.e. 65.71% and lower survival percentage was reported in *Citrus reticulata* Blanco cv. Nagpur mandarin i.e. 40.90% (Table 3 and Fig. 2).

This investigation underscores the efficacy of embryo culture as a robust biotechnological strategy for achieving uniform and high-frequency germination across diverse *Citrus* taxa. Our results identify a critical biochemical threshold for exogenous cytokinin supplementation; specifically, Murashige and Skoog (MS) medium enriched with 2 mg/L BAP consistently optimized the morphogenetic response across all evaluated species. Conversely, the transition to a supra-optimal concentration of 3 mg/L BAP resulted in an inhibitory effect, highlighting a narrow window for effective hormonal signaling in *Citrus* embryonic tissues.

Beyond chemical optimization, the physiological status of the explant emerged as a decisive factor. A strong positive correlation was established between embryo size and regenerative success, with larger embryos (7.0–11.0 mm) exhibiting superior germination rates–reaching a peak of 98.46% in *Citrus limon*. This enhanced performance is likely attributed to the more substantial nutrient reserves and a more balanced endogenous phytohormone profile present in advanced developmental stages.

While *C. limon* and *C. reticulata* cv. Kinnow demonstrated high plasticity and survival, the lower

Table 3: Survival and acclimatizationa of *In Vitro* derived plantlets.

Sr. no.	Citrus species	Embryos used for culturing	No. of plants transferred	No. of plants survived	Survival percentage (%)
1	*Citrus aurantifolia*	E	20	11	55
2	*Citrus limon*	E	35	23	65.71
3	*Citrus sinensis*	E	22	13	59.09
4	*Citrus reticulata* cv. Kinnow	E	30	19	63.33
5	*Citrus reticulata* Blanco cv. Nagpur mandarin	E	22	9	40.90

Fig. 2. Embryos transferred in soil for acclimatization

response observed in *C. reticulata* cv. Nagpur mandarin suggests that genotype-specific recalcitrance remains a challenge. Ultimately, the optimized protocols developed here for Kinnow and Nagpur Mandarin offer a validated framework for embryo rescue, providing a vital tool for triploid breeding and the accelerated development of seedless, high-quality citrus varieties.

AUTHOR'S CONTRIBUTIONS

Conceptualization of research (S); Designing of the experiments (AK); Contribution of experimental materials (S,AK); Analysis of data and interpretation (S,AK); Preparation of manuscript and literature review (S,AK).

DECLARATION

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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