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Elucidating the effect of plant bioregulators on embryo maturation for shortening the breeding cycle in papaya

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ABSTRACT

Papaya (*Carica papaya* L.) is a tropical fruit crop with commercial importance due to its nutritional and medicinal value. The long generation time required by papaya plants during the breeding process significantly slows crop improvement, and developing a variety takes 15-16 years (7-8 generations). As a solution in this direction, using plant bioregulators (PGR) to improve seed maturity in conjunction with the embryo culture technique may aid in reducing the time between fruit set and seedling establishment. We investigated the effect of ethrel, abscisic acid (ABA), and methyl jasmonate (MeJa) in different concentrations during early fruit development on hastening embryo maturity followed by embryo culture for shortening the breeding cycle of dioecious papaya var. Pusa Nanha. Fruits after 60 and 75-days old fruit (DOF) were treated with ethrel (100, 120 ppm), abscisic acid (10^{-6}, 10^{-4} M), and MeJA (10^{-5}, 10^{-3} M); the effect of treatments was observed after 15 days of PGR application. Among the treatments, ethrel 120 and 100 ppm at 75 DOF were found to be effective for hastening seed maturity, as measured by seed colour (light brown), embryo formation (85%), and embryo size (2.93 mm), maximum *in vitro* germination (85%), rapid shoot emergence (5.67 days), earliest radicle emergence (2.67 days), resulting in maximum plantlet regeneration (86.67%). The embryos isolated from the fruits treated with MeJA (10^{-5} M) showed the maximum number of roots (5.13), with 75.50 % plantlet regeneration. As a result, using the above method, the breeding cycle of papaya could be reduced to as low as 3 months, compared to the standard period of 6-9 months (from pollination to seedling establishment).

Key words: *Carica Papaya* L., ABA, Ethrel, Embryo culture MeJA,

INTRODUCTION

Papaya is India's 4th most important fruit crop and is grown commercially in the tropical and subtropical regions of the world under different names, like *papita*, pawpaw and tree melon (Ram, 10). India is the world's largest producer of papaya, followed by Brazil, Mexico and Nigeria. Worldwide, 13.158 million MT of papaya is produced in about 4.6 lakh ha (FAO, 2). Papaya is a highly cross-pollinated perennial crop, and for varietal improvement, approximately 7-8 generations (15-16 years) are needed to release a variety (Ray, 12). The growth cycle of papaya is completed in three phases: seed sowing to seedling rearing (1.5 to 2.0 months), planting to flowering (4-5 months) and fruit setting to fruit harvesting (4-6 months). Thus, shortening the papaya growth cycle seems important for efficient breeding (Tamaki *et al*., 14). The period from fruit set to fruit harvest may be shortened by culturing embryos *in vitro* (Fig. 1). Papaya embryo culture has been applied to produce inter-specific hybrids through embryo rescue. Distant crosses in papaya typically fail due to embryo abortion 70-90 days after pollination, and hybrid embryos must be cultured *in vitro* to be rescued. Hence, producing such hybrids from cross-incompatible species embryo culture and embryo rescue is vital in papaya crop improvement.

The use of plant bioregulators has many applications in the horticulture industry. Ethylene is one of the phytohormones with many roles in plant growth and differentiation. Ethylene has many functions in plant tissue cultures' organogenesis, embryo germination, and somatic embryogenesis. Similarly, abscisic acid is an essential plant bioregulator for embryo development and maturation. Jasmonates (JAs), including jasmonic acid and its methyl ester, are lipid-derived chemicals that have signalling roles in plant growth and development and stress responses. As natural plant bioregulators, JAs are found in abundance in plants. Recently, research has revealed that the use of JAs appears promising at different stages of the micro-propagation of various species. Under *in vitro* conditions, MeJA increases the

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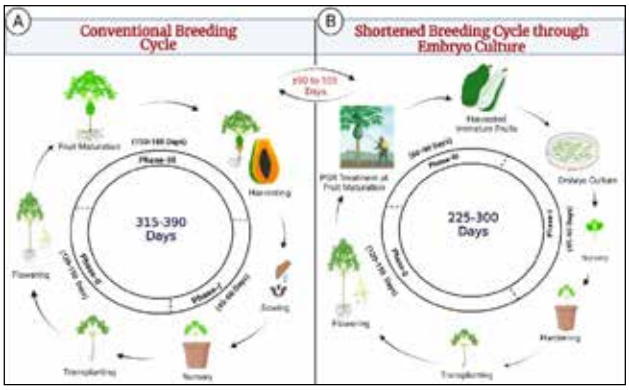

Fig. 1. (A) Conventional breeding cycle of papaya completed within 315-390 days. (B) Shortened papaya breeding cycle via embryo culture, completed 90-105 days earlier than the conventional breeding cycle (Picture created through Biorender.com).

proliferation rate of shoots, roots and callus (Kaminska *et al*., 7). However, there are very few reports on the hastening of embryo maturity in papaya using ABA and MeJA. Therefore, the present investigation has been undertaken on the effect of ethylene, abscisic acid and methyl jasmonate on *in vivo* embryo maturation and, ultimately, *in vitro* regeneration.

MATERIALS AND METHODS

The plants of Pusa Nanha were maintained at Horticulture Farm of the Division of Fruits and Horticultural Technology, ICAR-Indian Agricultural Research Institute, New Delhi, located at 77°12'E longitude, 28°40'N latitude, and an altitude of 228.6 m above mean sea level. Seeds of papaya var. Pusa Nanha were germinated in portrays following the recommended practice. Seedlings were transplanted in the field at 1.5 × 1.5 m^2 apart in a square system. During the peak pollination period, i.e., in September, selfing or sib-mating was carried out during flowering to get 60- and 75-day-old fruits (DOF) for the experiment.

To prepare ethylene solution, 100 µl a.i. and 120 µl a.i ethrel were taken out and dissolved in one litre of double distilled water. For making ABA solutions, 26.4 and 0.264 mg ABA, respectively, in powdered extra pure form, was taken and dissolved in 10 ml of ethyl alcohol, and the final volume was made up by adding distilled water. For the preparation of MeJA solutions, 224 and 2.24 µl MeJA in pure liquid form was accurately measured and dissolved in 10 ml of ethyl alcohol (99.99% v/v) and the final volume was made up of one litre of double-distilled water.

Individual fruits of 60-75 days old were bagged using 30x30cm polyethylene pouches containg 50 ml ethrel. The whole pouch was covered by aluminium foil to maintain uniform temperature. After 15 days the both the aluminium foil and the polyethylene bags were removed. Similar procedures were followed for treatment with abscisic acid and MeJA treatments. Poly bags containing only distilled water served as control. The fruits were harvested on the 15th day for further studies. A semisolid media containing Murashige and Skoog (MS) salts (Hi-media®) supplemented with BAP (0.01 µM; Sigma Aldrich), sucrose (3%; Hi-media®) and agar (0.8%; Qualigens, Mumbai) was used for the embryo culture. Each fruit was surface-sterilized using a flame sterilization technique. Immature papaya fruit were cut longitudinally with a knife under sterile conditions. The seeds were dissected and inoculated onto petriplates containing embryo culture medium. The petriplates were then shifted to the growth chamber under standard culture room conditions (Temp.: 22 ±2°C; 16/8 h light and dark cycle). Pictures of embryos (Fig. 3) were taken under a stereo zoom microscope (SZM-167, LMI, UK) using the software MvImage Ver. 5.0.

Based on daily visual observation, embryo germination was counted based on radicle size > 5.00 mm (Tamaki *et al*., 14). The days taken for radicle emergence were observed daily, where the first three germinated embryos were counted as the date of radicle emergence. Based on daily visual observations, the days taken for the first three emerging shoots (> 5.00 mm) were considered the date of shoot emergence. For observation of average plantlet weight after 30 days of embryo inoculation, each plantlet's weight was measured accurately with the help of an electronic weighing machine and expressed in milligram (mg). For counting the total number of primary roots per plant, an average of three plantlets was recorded from each treatment after 30 days of embryo inoculation. After 30 days of embryo inoculation, the plantlets were removed from each petriplate, and five roots were measured and expressed in centimetres using a scale (cm). Plants with a well-developed shoot (> 20 mm length), roots (> 10 mm length), and more than 20 mg weight were considered regenerated plants and transferred into pro-trays after 30 days of embryo inoculation. The regeneration percentage was calculated by dividing the total number of regenerated plantlets by the total number of embryos inoculated. The research data for various parameters were statistically analyzed using Factorial Randomized Block Design using SAS software, with P-values 0.05 considered statistically significant.

RESULTS AND DISCUSSION

In the present study, it was found that MeJA and ethrel both significantly increased embryo size (Fig. 2). The present investigation supports the previous finding that embryos in avocado fruits that were exogenously treated with ethylene gas grew

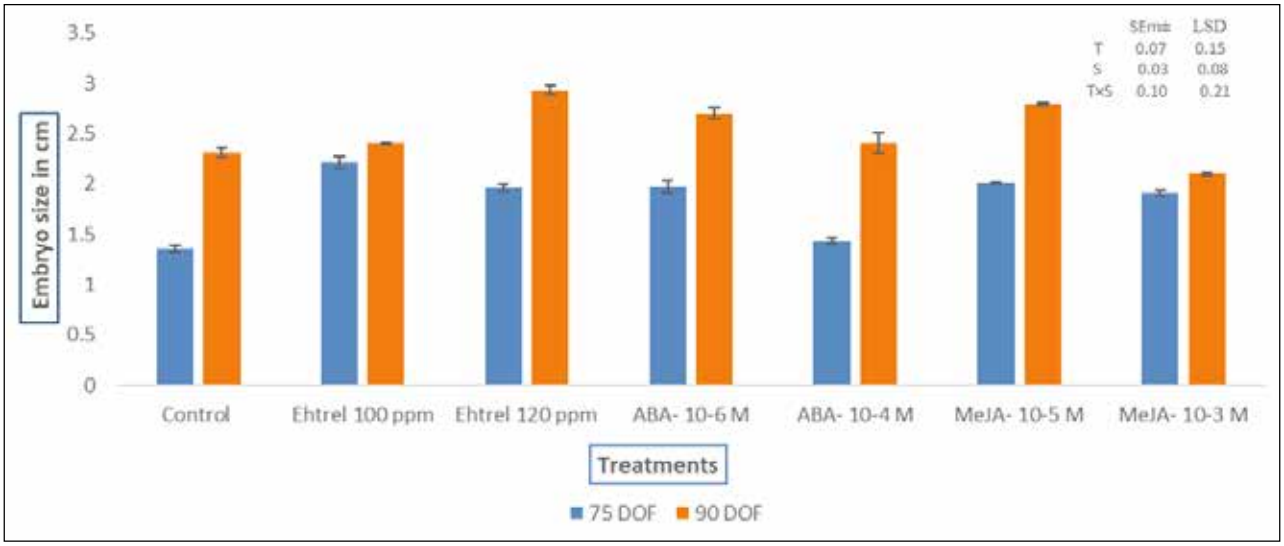

Fig. 2. Effect of ethylene, ABA and MeJA on embryo length (grey & yellow bar) in papaya. (where T-treatment, S- stage).

longer than those in untreated fruits, indicating that ethylene is not required for germination but can considerably enhance embryo size (Hershkovitz *et al*., 4). A similar finding was also reported in Damson Plum (Fernandez-Otero *et al*., 3).

Seeds are the most economical part of papaya fruit. Under natural conditions, the colour of the papaya seed coat changes from white to brown after 90 days of flowering, then to black approximately 114–140 days after flowering. In the present investigation, 60plant bioregulator- and 75-day-old immature fruits were treated with different plant bioregulators, and no significant changes were observed in the seed coat colour (Fig. 3). The growth of seeds and fruits are strongly related and synchronized. It is now well accepted that seeds contain a rich supply of hormones, notably auxins, GAs, and cytokinin, which are essential in promoting surrounding tissue growth and even determining the size of the fruit. Gibberellins (GAs) are synthesized in immature seeds, and as the fruit matures, the fruit begins to generate ethylene, which causes the seed coat to change colour (Ying and Sim, 15). As a result, the seeds extracted from the untreated immature fruits showed no change in colour. Only ethrel treated fruits had light brown seeds (Fig. 3). A similar finding was observed in *Brassica,* where ethylene production was high in developing embryos when embryos began to degreen during maturation (Johnson-Flanagan and Spencer, 6). During embryo extraction, the total number of embryos were counted with reference to the total number of seed taken and expressed in percentage (%). In young fruit, fewer embryos were isolated; with maturity, the number of embryos isolated (per 30 seeds) increased. Ethrel and MeJA, respectively, promoted embryo development, and in the study, control fruits were found to be ineffective. Embryo development was highest in 90 DOF treated with ethrel 100 ppm (79.72%), followed by ehtrel 120 ppm (77.80%) and lowest in 75 DOF control fruits (34.98%; Fig. 4). Ethrel was found to be the best of all the treatments. In damson plum, ethylene production increases early in the seed's

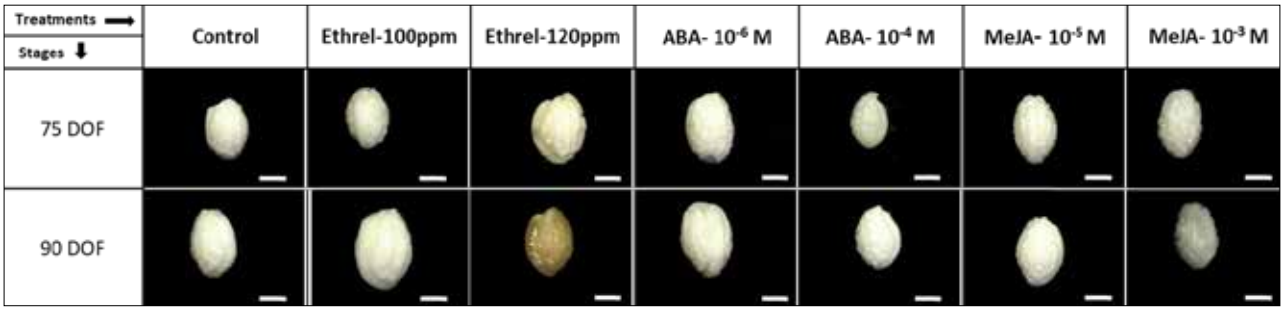

Fig. 3. Effect of ethylene, ABA and MeJA on seed coat colour of immature seeds of papaya. Measurement of seed size and at 0.8 X taken under a stereo zoom microscope.

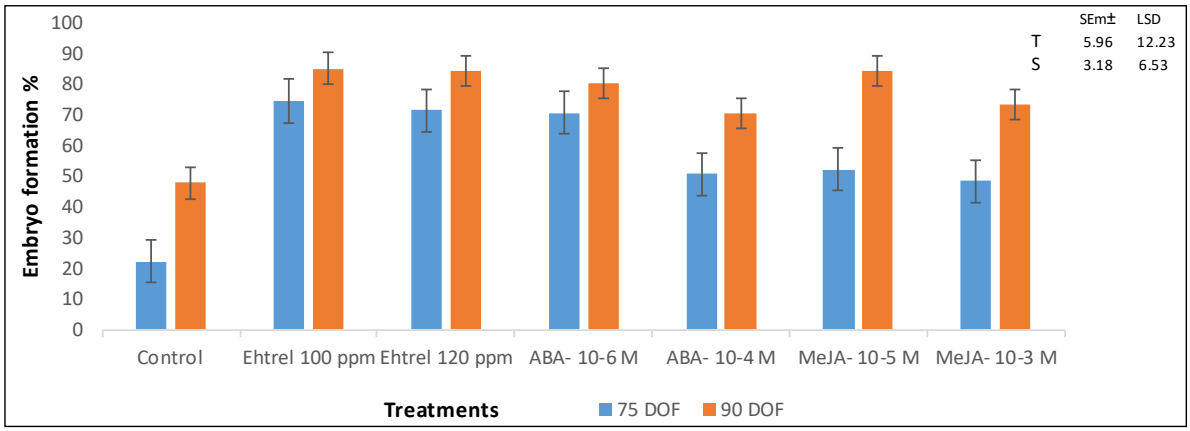

Fig. 4. Effect of ethylene, ABA and MeJA on per cent embryo formation in papaya presented in bar diagram.

growth and reduces later in the seed's development (Fernandez-Otero *et al.*, 3). Sano and Marion-Poll (13) also observed that ABA inhibits embryo growth and development during the seed maturation phase.

During embryo germination, the total number of germinated embryos was counted with reference to the total number of embryos inoculated and expressed in percentage (%). In the present investigation, the highest percentage of embryo germination was found in the ethrel (97.76%) treated fruit and the lowest in control (23.34%; Fig. 5). Tamaki *et al*. (14) found a similar response in ethrel treated 85 days old fruit of papaya, where embryo germination was substantially higher in the treated fruit than that in control. Exogenous ethylene treatment has been shown to promote embryo germination in apples (Kepczynski *et al*., 8). Apart from ethrel, a lower concentration of MeJA 10^{-5} M (81.67%) was better than that of MeJA 10^{-3} M (61.10%). JA facilitated the emergence of dormant *Malus* embryos (Ranjan *et al*., 11).

Juvenile explants, such as immature zygotic embryos, have a high generation potential (Ikeuchi *et al.,* 5). In 90 DOF, ethrel and the lower concentration of ABA-treated fruits showed precocious emergence

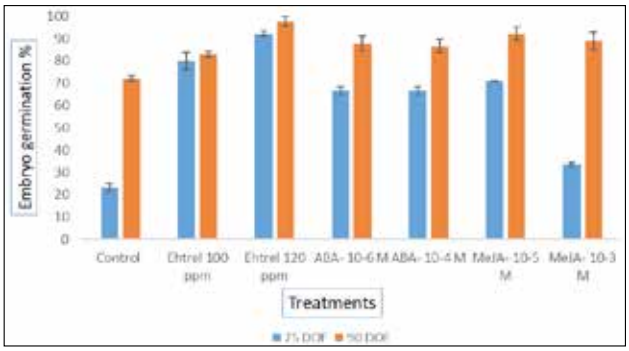

Fig. 5. Effect of ethylene, ABA and MeJA on embryo germination (%) in papaya.

of radicles, better than the higher concentration of ABA, MeJA and control. Among ethylene, 100 ppm showed more rapid radicle growth (2.67 days) than a higher concentration (120 ppm) of ethrel (3.67 days; Fig. 6). Abts *et al.* (1) observed the same response to ethylene in sugar beet: a minor increase in ethylene production (10 µM ACC) causes an increase in root elongation, whereas a sharp rise in ethylene production causes a decrease in root elongation. Naidu and Sreenivasan (9) studied the effect of abscisic acid on cultured zygotic embryos of *Coffea arabica,* supported the present finding and reported that ABA concentrations increased from 0.4 to 18.9 µM reduced the embryo growth.

In the present study, embryonic growth followed by rapid shoot emergence was observed in ethrel (100 & 120 ppm) followed by ABA (10^{-6} M) treated fruits, while the slowest shoot growth was found in control (Fig.6). This is also well known that ethylene improved the elongation of shoots and roots in aquatic plants. The present study also confirmed that ethylene played a role in mediating ABA's impact on embryogenesis, and it has been found that exogenous ABA enhances ethylene biosynthesis. In the present study, the effect of MeJA was not significant.

The culture time between 20 and 30 days seems necessary for plant growth and subsequent root formation. During this phase, cell division is active, resulting in the formation and expansion of the initial buds and the induction of new bud and root primordia. Our findings demonstrated that methyl jasmonate and ethylene regulate morphogenesis in the *in vitro* grown *Carica papaya* immature embryos and found the maximum number of roots per plant in MeJA 10^{-5} M (3.67) and ethrel 100 ppm (3.67), whereas embryos isolated from ABA 10^{-4} M (2.43) treated fruits showed a minimum number of roots per plant (Table 1). In *Solanum tuberosum*, Zhang *et al.* (16) found that 9.5

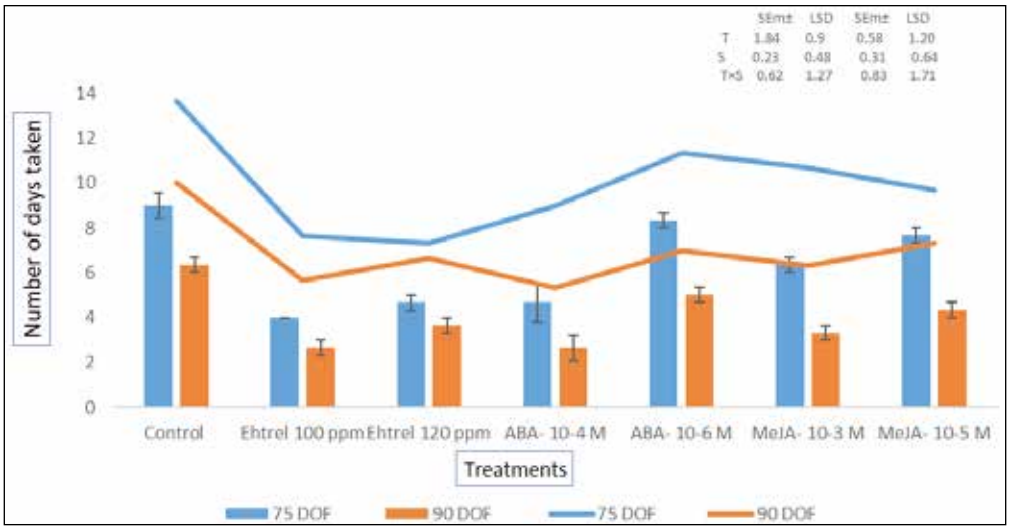

Fig. 6. Effect of ethylene, ABA and MeJA on total number of days taken for radicle emergence (bar diagram) and shoot emergence (line diagram) in papaya.

µM JA increased the fresh weight of the shoot and root, the number of roots; however, more than 9.5 µM JA inhibited the root growth.

In the present investigation, 90 DOF (67.30%) was found to be more significant than 75 DOF (32.03%) and showed a relatively good plantlet regeneration percentage (Table 1). The fresh plantlet weight increased in ethrel-treated fruits, followed by ABA, MeJA and control (Fig.7). These results indicated that ethylene enhances the growth of papaya seedlings produced from embryos isolated from immature fruits treated with ethrel. Tamaki *et al*. (14) observed a similar response of ethrel on papaya immature zygotic embryos.

The findings of this study pave the way for shortening the breeding cycle for papaya, which will prove to be of immense significance for breeders and help in their efforts to evolve new varieties faster. Under the north Indian conditions, papaya var. 'Pusa Nanha', when exogenously treated with 120 and 100 ppm ethrel, improved the embryo and seed maturity, followed by 10^{-5} M MeJA. Based on the findings, we concluded that *in vitro* embryo culture

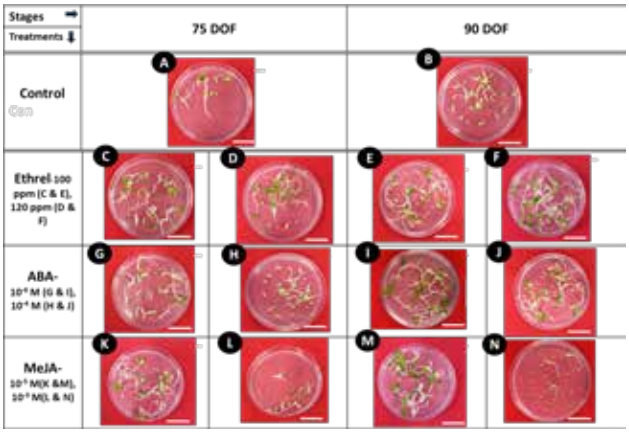

Fig. 7. Effect of ethrel, ABA and MeJA on plantlet regeneration. (In all images A to N *bar* = 1.5 cm.

Table 1. Effect of ethylene, ABA and methyl jasmonate on number of roots and plantlet regeneration.

Treatment	No. of roots per plantlet		Plantlet regeneration (%)	
	75 DOF	90 DOF	75 DOF	90 DOF
Control	2.13 ± 0.13^{f}	3.55 ± 0.07^{c}	12.23 ± 1.11^{e}	51.10 ± 1.11^{c}
Ethrel-100 ppm	3.13 ± 0.06^{d}	4.20 ± 0.11^{b}	50.00 ± 1.92^{c}	71.10 ± 4.44^{b}
Ethrel-120 ppm	3.53 ± 0.18^{c}	3.70 ± 0.20^{c}	67.67 ± 1.92^{b}	86.67 ± 3.85^{a}
ABA- 10^{-6} M	2.13 ± 1.33^{f}	3.80 ± 0.10^{c}	44.43 ± 1.11^{c}	71.10 ± 1.11^{b}
ABA- 10^{-4} M	2.13 ± 1.33^{f}	2.73 ± 0.12^{e}	25.33 ± 1.11^{d}	44..43 ± 2.93^{c}
MeJA- 10^{-5} M	2.20 ± 0.10^{f}	5.13 ± 0.12^{a}	14.43 ± 1.11^{e}	75.50 ± 1.95^{a}
MeJA- 10^{-3} M	2.20 ± 0.10^{f}	4.30 ± 0.12^{b}	11.10 ± 1.11^{e}	63..33 ± 5.77^{b}
Mean	2.50^{b}	3.91^{a}	32.03^{b}	67.30^{a}
	SEm±	LSD	SEm±	LSD
Treatment (T)	0.13	0.27	2.67	5.50
Stages (S)	0.07	0.15	1.40	2.93
T × S	0.19	0.39	3.80	7.67

with good-quality embryos enhanced by MeJA and ethrel treatments could shorten the breeding cycle of papaya by approximately 3 to 4 months. It is further suggested that 10^{-5} M MeJA be utilized for improving root formation, plantlet growth, and subsequent regeneration *in vitro*. Further detailed research is required to fully understand the biochemical mechanisms involved in the seeds due to the effect of these phytohormone applications to fully tap the potential in different crops for hastening maturity and improving fruit quality parameters.

AUTHORS' CONTRIBUTION

Conceptualization of research (KS and JP); Designing of the experiments (KS and JP); Contribution of experimental materials (KS and VS); Execution of field/lab experiments and data collection (BS); Analysis of data and interpretation (BS and BKY); Preparation of the manuscript (BS, VS and VM).

DECLARATION

The authors declare that there is no conflict of interest.

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