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**RESEARCH ARTICLE**

# BREAKING EMBRYONIC SEED DORMANCY OF *MALUS PUMILA* MILL. (APPLE) UNDER *IN-VITRO* CONDITIONS BY KINETIN

*** Yatoo, Ghulam Mohiuddin and Nathar, Varsha Nitin**

Department of Botany, S. G. B. Amravati University, Amravati- 444602 (Maharashtra) India

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**ABSTRACT**

Mature seeds of apple (*Mallus pumila)* Mill. (Cv. Golden delicious) are dormant and do not germinate unless their dormancy is removed by several weeks of moist-cold treatment. We investigated the effect of short-term growth regulator (NAA, BAP, Kinetin and 2,4-D) pre-treatment on breaking of apple embryonic dormancy. Embryos excised aseptically from non stratified Cultivator 'Red Delicious' apple seeds were placed in a solution of various growth regulators at different concentration for different time period before germination in Murashige and Skoog's media in order to remove embryonic dormancy in them. It was observed that only Kinetin pre-treatment at lower concentrations (5, 10, 15 and 20 mg/l for 6-12 hours) proved to break embryonic dormancy of apple. At higher concentration of Kinetin pre-treatment i.e. 40, 60, 80 and 100 mg/l for the time period of 2-4 hours, the apple embryos were transformed to callus. The germinated apple plantlets after one month of germination were hardened in plastic cups contained sterile garden soil, farmyard soil and sand (2:1:1) and finally transferred to green house. The plantlets showed 90 % survival rate. The objective of this study was to remove embryonic dormancy of apple seeds by various growth regulator pre-treatements and enhance germination in a shorter period of time instead of going for stratification for several weeks. This could be very useful in plant breeding programme by shortening the breeding cycle and introducing the apple to tropical regions where prolonged winters are not observed.



## INTRODUCTION

Apple (*Malus pumila*) is the most important temperate fruit of the north-western Himalayan region. It is predominantly grown in Jammu and Kashmir, Himachal Pradesh and hills of Uttar Pradesh. Apple, the pomaceous fruit is perennial and most widely cultivated fruit tree. The genus- *Malus* Mill.- includes 25 species of deciduous trees or shrubs native to the temperate region of North America, Europe and Asia. Germination may be defined as the process associated with the initiation and completion of embryo emergence; it refers to the progress of a seed from imbibition through radical emergence (Bewley and Black 1978; Nonogaki *et al*., 2007). Germination in higher plants is under the control of a combination of internal and environmental factors. The conditions affecting germination include temperature, moisture and the availability of oxygen. In many cases germination may also depend on the quantity and quality of light. The seeds of some plants also show dormancy. This reflects the presence of an internal block to germination even when external conditions are favourable for germination. The block is removed by pre-treatment such as a period of after-ripening, chilling or a brief exposure to light (Bewley and Black, 1982). Dormancy in apple seeds is brought about by several barriers that inhibit processes cardinal for germination. Some of them are of a structural character; the seed coat hampers water access to the embryo, thus affecting the imbibition and as a result, hampers the function of many enzymes, membranes and cellular organelles. The same structure limits the entrance of oxygen to the embryo, affecting the oxidative processes. Other barriers are of developmental character; dormant embryo mitochondria need maturation before they reach full activity. The best studied are, however, the metabolic barriers that render successful germination impossible. The dormancy in apple seeds is well expressed, its embryonic dormancy is completely removed by several weeks of cold stratification. Apple embryos isolated from dormant seeds germinate but the germination is markedly slower than that of stratified seeds and seedlings developing from such embryos are characterised by morphological anomalies.

The mature seeds of apple are dormant. Inability of these seeds to germinate is due to the dormancy of embryo as well as to the inhibitory effects of the seed coat. After removal of seed coat, dormant embryos germinate slowly and germination is stimulated by light and gibberellins or cytokinin treatments (Smolen´ska and Lewak, 1971). The most frequently postulated mechanism for control of embryonic dormancy is a block or a series of blocks in reserve mobilisation (Lewak, 1981; Dawidowicz-Grzegorzewska, 1989). ). Blocks in the mobilisation of storage lipids (Z_arska-Maciejewska and Lewak, 1976; Z_arska-Maciejewska, 1992), proteins (Dawidowicz-Grzegorzewska and Z_arska-Maciejewska, 1979; Z_arska-Maciejewska and Lewak, 1983) and

***Corresponding author:*** yatoobotany@gmail.com

oligosaccharides (Bogatek and Lewak, 1988) were demonstrated for dormant apple embryos.To accelerate breaking of seed dormancy, hormones have been applied in several studies (Keshtkar *et al.,* 2008). Plant growth regulators such as GA, IAA, IBA, Kintein and mechanical scarification such as hot water have been recommended to break dormancy and enhance germination (Hermansen *et al*., 1999). Synthetic CKs (kinetine and benzyladenine) stimulated germination of dormant embryos (Zhang and Lespinasse, 1991) and of embryos stratified no longer than for 30 days, similar to GAs (Lewak and Bryzek 1974; Lewak 1980). Cytokinins release the dormancy of seeds presumably by counteracting the effects of inhibitors by an as yet unknown mechanism (Khan, 1971). Apple seeds display dormancy which has been overcome by cold stratification. A minimum of about 30 days under stratification conditions is required to remove embryonic dormancy of apple (Zhang and Lespinasse, 1991). Apple seeds are easily available, convenient to handle in large scale experiments, and their manipulation to isolate embryos and their parts is fairly straight forward. For these reasons, apple seeds have been utilized for decades for studies of different aspects of dormancy in different research centres all over the world in the temperate zones. The present study was conducted to break embryonic dormancy and enhance the germination of apple embryos.

## MATERIAL AND METHODS

Experimental studies were carried out on apple seeds in the Plant Tissue Culture Laboratory, Department of Botany, Sant Gadge Baba Amravati University, Amravati (M.S.).

### Seed source

The seeds of apple cv. 'Red Delicious' were obtained in the month of September 2009 from Teng apple garden, village-Yadipora Palhallan, Kashmir where apple trees grow abundantly.

### Germination test

The seeds were stored at 35±2 $^{o}$C for three weeks before germination in order to induce dormancy in them. Then germination test was carried out to check their dormancy. The seeds were washed thoroughly with distilled water several times, rinsed with 70% alcohol for 5 minutes; surface sterilized with 0.1% mercuric chloride and finally washed with sterile distilled water to remove traces of mercuric chloride. The seeds were then placed aseptically on sterile culture bottles containing 40 ml of sterile plain Murashige and Skoog (MS) media, then seed coat was removed aseptically from apple embryos and then placed on MS media devoid of growth regulator concentrations. At last embryos of the seeds were removed aseptically and inoculated on plain MS media to check their dormancy.

### Growth regulator treatment

The growth regulators employed throughout this study to break embryonic dormancy and improve germination were 2,4-D, BAP, NAA and kinetin. The embryos of apple were removed aseptically, washed with sterile distilled water several times; surface sterilized with 0.1 % mercuric chloride for 2 minutes and finally washed again with sterile distilled water. The embryos were placed in different growth regulator solutions at different concentrations for different time period prior to inoculation in MS media (Table 1). The cultures were maintained under aseptic conditions at 20 ± 2 $^{o}$C with 16 hours light period.

**Table 1. Treatment of various growth regulators to aseptically excised apple embryos at different concentrations for different time period prior to germination in MS media**

| Growth Regulator | Concentration (mg/l) | Treatment time period (hours) |
|---|---|---|
| NAA | 5 | 6-12 |
| | 10 | 6-12 |
| BAP | 15 | 6-12 |
| | 20 | 6-12 |
| Kinetin | 40 | 2-4 |
| | 60 | 2-4 |
| 2,4-D | 80 | 2-4 |
| | 100 | 2-4 |

The concentration of each growth regulator varies from 5-100 mg/l.

### Acclimatization and transfer of plantlets to soil

Aseptic plantlets of apple were isolated from the culture media and washed with sterile double distilled water to remove adhering medium. These plantlets were transferred to plastic cups contained sterile garden soil, farmyard soil and sand (2:1:1). The primary hardening was carried out in programmable environmental chamber for 4 weeks. The temperature and humidity was adjusted to 20 ± 2 $^{o}$C and 50% respectively. The temperature of programmable environmental chamber was raised to 1 $^{o}$C for every 3 days. The plantlets were then transferred to normal laboratory conditions for 2 weeks. Finally after 43$^{th}$day, the plantlets were transplanted to Departmental Botanical Garden and placed under shade for further growth and development. The morphological, growth characteristics and survival efficiency was encountered.

**Table 2. Percentage germination of apple embryos on MS media after treatment with different kinetin concentrations for different time period.**

| Growth Regulator | Concentration (mg/l) | Time period (hours) | Germination (%) |
|---|---|---|---|
| | 5 | 6-12 | 90 |
| | 10 | 6-12 | 90 |
| | 15 | 6-12 | 80 |
| | 20 | 6-12 | 50 |
| | 40 | 2-4 | callus formation |
| Kinetin | 60 | 2-4 | callus formation |
| | 80 | 2-4 | callus formation |
| | 100 | 2-4 | callus formation |

Each value is the mean of 3 replicates.

## RESULTS AND DISCUSSION

The results showed that apple seeds with and without seed coat as well as aseptically excised embryos prior to various growth regulator pre-treatements, were not able to germinate on MS media even for two months as they were in the state of embryonic dormancy. Only excised embryos of apple after pre-treatement with various concentrations of kinetin were able to germinate. After treating large number of apple embryos with various concentrations of growth regulators for different time period, it was observed that only kinetin at lower concentrations i.e. 5, 10, 15 and 20 mg/l for 6-12 hours had positive effect on the germination, while other growth regulators like NAA, BAP and 2,4-D were not able to break

dormancy (Table 2). Exogenous 1-14C IAA, taken up by isolated apple embryos, was found to be rapidly converted into conjugates with aspartate and short peptides containing an aspartate moiety (Dziewanowska and Lewak 1987). These observations indicate that auxin is not involved in the removal of apple embryonic dormancy. Kinetin at higher concentration i.e. 40, 60, 80 and 100 mg/l for the time period of 2-4 hours transformed the apple embryos into callus (Table 2). The highest germination percentage i.e. 90 occured after treating embryos with 5 mg/l (Fig.1, A) and 10 mg/l kinetin for 6-12 hours. Yong Xiang Zhang and Yves Lespinasse (1990) were able to remove embryonic dormancy in apple by 6-benzylaminopurine (BAP) at 12.5 to 25 mg/l for 6-12 hours. The germination percentage was 80 (Fig. 1, B) at 15 mg/l kinetin treatment for 6-12 hours, while it was 50 % at 20 mg/l of kinetin for 6-12 hours (Fig. 2).

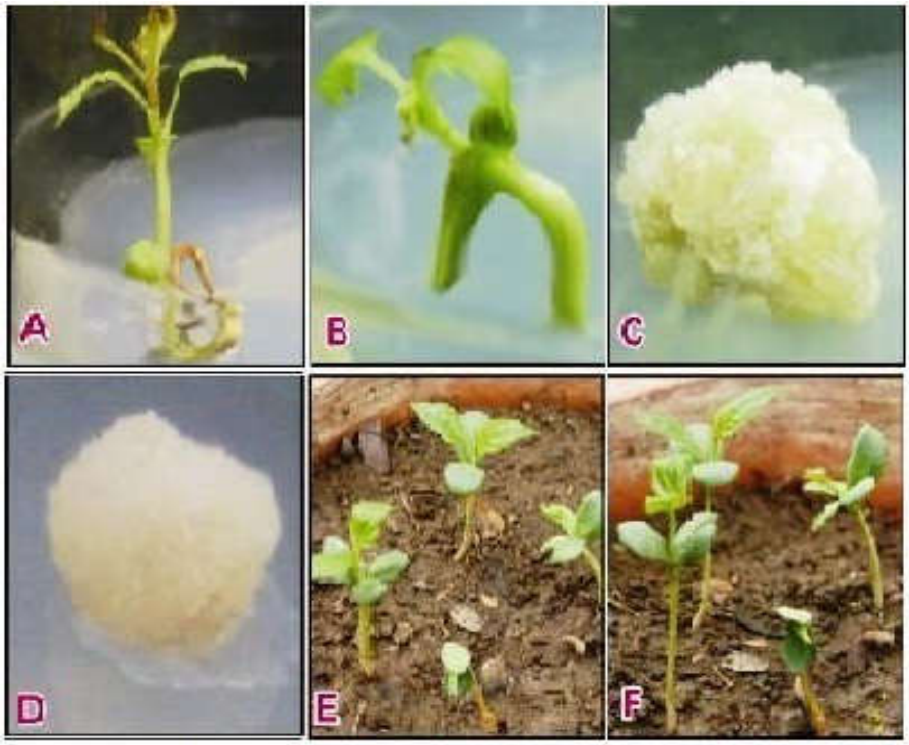


**Figure 1: In- Vitro germination studies of Malus pumila on MS media.**

**A: Germination of apple seed embryo after pre-treatment with 5 mg/l kinetin for 6-12 hours; B: Germination of apple seed embryo after pre-treatment with 15 mg/l kinetin for 6-12 hours; C & D: Callus formation from apple seed embryo after pre-treatment with 60 and 100 mg/l kinetin for 2-5 hours: E & F: Acclimatized apple plants.**

The embryos were transformed into callus at 40 mg/l, 60mg/l and 80 mg/l (Fig. 1, C) and 100 mg/l (Fig. 1, D) kinetin treatment. The callogenic response was very low, the callus formed was firm, dense, non embryogenic and whitish in colour. Kirdar and Ertekin (2001) reported that polystimulin A6 (PS-A6) and polystumilin K (PS-K) with 100 ppm concentrations furnish the best results on seed germination in *Magnolia grandiflora*. Rao *et al.,* in 1975 demonstrated that kinetin is capable of overcoming the inhibition of germination in Grand Rapids lettuce seeds by high temperature. Breakage of apple embryo dormancy is also observed in the presence of gibberellic acid (GA) (Smolejska and Lewak, 1971) or after HCN (1 mM, 6 h) pretreatment (Bogatek *et al.* 1991). Cytokinins have been reported to cause expansion of cotyledons (Letham and Williams, 1969). An increase in the percentage of polyribosomes by kinetin and ethrel is presumably due to an increase in mRNA-ribosome attachment. The present study reveals that at lower concentration of kinetin pre-treatement, seed germination occurs while as the concentration of kinetin was increased, there was decrease in seed germination and at higher concentration of kinetin pre-treatement callus formation occurs.

Thus it can be suggested that kinetin pre-treatment at lower concentration upto 20 mg/l is suitable for seed germination. Thus the seeds of many plant species which display dormancy over a long period of time can be overcomed by germinating these seeds with kinetin pre-tratement under aseptic conditions. Germinated plantlets of apple were isolated from the culture media and washed with sterile double distilled water to remove adhering medium. These plantlets were transferred to plastic cups that contained sterile garden soil, farmyard soil and sand (2:1:1). The survival rate after hardening for first 2 weeks was 100%. However it decreased to 90% after 6 weeks of acclimatization. There was no detectable variation among the acclimatized plants with

respect to morphological and growth characteristics. All of the germinated plants were free from external defects (Fig. 1, E & F) and are maintained in Departmental garden.

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