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Optimizing of Synthetic Seed of *Malling apple (Malusdomestica)* Rootstocks cv. M26 and cv. MM106

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Abstract: Malling Increasing demands for apple across the world requires its rapid production. Agricultural biotechnology has met this need and has prevented some problems during adolescence period and long generation. We investigated embryonic and non-embryonic callus through cultivating apical and lateral buds on different treatments with MS base culture and BAP (0-2.5 mg/l) and IBA (0-5 mg/l) hormones Kin and NAA hormones. The highest percent of embryogenesis related to T_{11} treatment (IBA 4 mg/l, BAP 1 mg/l) and T_{23} (IBA 4 mg/l, BAP 2.5 mg/l). The embryos were in corpuscular stage. Then third sub-culture was performed and all calluses and embryos were delivered to their corresponding treatments. K_5 (NAA 1 mg/l, Kin 0.5 mg/l) and K8 (NAA 3 mg/l, Kin 1 mg/l) yielded the highest amount of callus (5.3 mm). The embryos remained in corpuscular stage and they did not show reproductive ability and developmental process. After preparation of artificial seed no regeneration was seen. By embryogenesis of MM.106 and M.26, the purpose of this research is improving a protocol for generation of two somatic cultivars.

Keywords: Malling, Synthetic Seed, Tissue Culture

1. Introduction

Increasing demands for apple across the world requires its rapid production. Agricultural biotechnology has met this need and has prevented some problems during adolescence period and long generation [20] Room (1994). Although biotechnology offers high capabilities in improvement of quality and production of apple, many apple cultivars resist reproduction through tissue culture [27] Jra-long Yao (1996). During working with the first description of somatic embryogenesis was independently obtained in carrot [11] Steward et al (1985). [22] Sharp (1982) and [25] Tisserat (1979) found that some parts of flower, immature embryo, flag, seeds, pollen are good materials for somatic embryo production.

Two types of somatic embryogenesis are recognized: Direct: embryo is produced directly from a cell or tissue, without callus formation. Indirect: embryo is formed from embryonic callus. [1] Ammirate (1986), [10] Kohlenbach (1985), [22] Sharpe et al. (1982) used cotyledons of immature zygotic embryos. Root and somatic embryos were formed. David James used immature seeds of four cultivars of apple. The embryos were obtained from endosperm in 50 days seeds at 5-23% in these four cultivars. In the reports of [14] Barbieri & Morini leaves is described. These factors include: nitrogen and its type, growth regulators, incubation conditions, leaf propagation and leaf maturation and the direction of explants [28] Yepez (1994). Some species have genotypes that don't produce seeds [6] Fasolo (1989) they have important factors in reproduction of apple cultivars through the seeds that they are healthy but they lack duplication power. These species are reproduced in vegetative manner in order to maintain their genotype. The plants obtained from tissue cultivation lack diseases, so they could be propagated and mass produced in all seasons.

By embryogenesis of MM. 106 and M. 26, the purpose of this research is improving a protocol for generation of two somatic cultivars.

1.1. Somatic Embryogenesis Induction

In natural conditions, differentiation pathway of plant cells depends on their later location and growth that can be easily

changed under in vitro conditions. Sever changes in surrounding environment of cells, such as putting the cells or tissues in artificial feeding environment or hormones may cause severe stress in cells. Investigations indicate that the more active, young and meristematic micro-samples tissue, the higher their ability to induce embryogenesis. Any change in the cell and directing it to embryogenesis requires a stress. Certainly, what must be considered after selecting the type of micro-sample for inducing embryogenesis in cells is presence of a hormonal agent and a stressor such as osmotic pressure, pH, temperature, light reduction and lesion thereby the plan of embryogenesis could be executed in micro-samples cells. Growth regulators applied externally have a main role in facilitation of morphologic changes required for somatic embryo production. From among growth regulators, auxines play significant role in inducing somatic embryogenesis. Also, D- 2, 4 as an artificial auxine is the most important auxine for somatic embryogenesis induction. Certain concentrations of this auxine solely or in combination to other auxine- based or non auxine- based growth regulators such as cytokinins are used for somatic embryo production. Auxines have the most impact on somatic embryogenesis induction. It is believed that auxine changes differentiation gene expression.

Somatic embryogenesis is a process in which somatic cells are converted into embryo. Somatic embryos are morphologically similar to reproductive embryos. Somatic embryos are bipolar and consisted of special embryotic organs, namely plumule, radicle, petiole, hypocotyl and cotyledons that result in embryos without vascular relation to maternal tissue. This process maybe ginusing the cells of different plant cells. For example the roots, petiole, hypocotyl and parenchyma of leaf maybe used as micro-samples for somatic embryogenesis in carrot.

1.2. Characteristics of Embryogenesis Cell

Embryo genetic cells possess meristem tic cells. They are divided rapidly; they have small size with dense cytoplasm, large nucleus, small vacuoles and numerous starch seeds. Histological and molecular studies confirm high RNA synthesis and metabolistic activity in these cells.

Callogenesis begins from 5th week, after callogenesis, explants containing callus and without callus were transferred to similar treatments where they were during first sub-culture. All plates containing explants were individually investigated in terms of embryo presence. Explants were sub-cultured 4 times and each sub-culture lasted one month and in each run they were investigated in terms of presence or absence of embryo. Some images were taken from calluses and embryos using stereo microscopes. In present study, no somatic embryo was obtained, so that preparation of artificial seed from somatic embryo is not possible.

2. Materials and Methods

2.1. Plant Materials, Chemicals

In this research, both apical bud and lateral bud were used for both cultivars, namely M.26 & MM. 106. We prepared sterilized explants of cvM.26 and CV MM.106 from agriculture research institute in Karaj city. All routine chemicals were prepared by lab stuff. Chemical materials had been purchased from Sigma.

2.1.1. Medium Preparation

Apple rootstocks of M.26 and MM.106 were maintained on MS (Murashige & skoog, 1962) medium consisting of macro and micro elements and supplemented with MS vitamins, different hormones such as IBA, 7.5 g l-1 agar and 30 g l-1 sucrose. The stock solution of all ingredients were stored under refrigeration.

2.1.2. Culture Conditions

The pH of media was adjusted to 5.8 before autoclaving at 121°C for 40min. All cultures were incubated under fluorescent lights with 16h lightness and the temperature was 25±2°C.

2.2. Experiment

First of all, sterile explants of two cultivars were cultured on MS treatment with BAP=2mg/l, IBA=0.1mg/l with 3 replications per treatments and 5 explant (shoot) per replication. Every treatment was cultured for 30 days. cultures were prepared in laminar air flow aseptically, after preparation, cultures were taken into incubator and after regeneration callus induction of Malling 26 and 106 was studied during three growth periods (30 days). If we observed callus in every treatment we fallowed embryo by a stereoscope.

For preparation of artificial seed at first, we prepared 0.1 liter sodium alginate solution 2% in MS environment and 0.1 litrecalcium solution 1% in distilled water. Then, we autoclaved these two solutions and all instruments including forceps, scalpel, distilled water, basket, magnet, pipet, syringe, etc.

All stages of artificial seeds preparation are performed under the hood and under sterile conditions. Sterilized shaker was located under the hood; calcium chloride must be rotating on shaker; apical and lateral buds of each cultivar were individually put in sodium alginate solution and using a syringe and pipet, alginate impregnated bud is put in rotating calcium chloride; the seeds wait in calcium chloride for 45 minutes to one hour and then we passed them through a filter and washed them with sterile distilled water for a few minutes. Obtained seeds were put in a sterile plate and covered them with foil and put them in refrigerator under 4°C temperature. For two months, the buds were investigated weekly in terms of regeneration.

2.3. Statistical Analysis

Analysis of the data was carried out by using Analysis of Variance (ANOVA) technique, SPSS and means were compared by using Least Significance Difference (LSD) Test at 5%probability level (Steel *et al*., 1997).

3. Results

3.1. Investigating Callus Production and Somatic Embryo in Apple, Malling Base, M26 and MM 106 Cultivars in Different Treatments During Subcultures

In present study, different environments for callus induction were prepared each one having different hormones and same vitamins and sucrose on leaf explants and lateral and apical buds of two cultivars. It seems that since apple is a wooden plant, its embryogenesis is not per formed rapidly. After locating explants on callus induction environments, sliced cells were stimulated and isolated and after isolation they began propagation the result of which was a small callus mass the diameter of which increased gradually. In present study, two types of callus were observed: embryonic callus in small number and non-embryonic callus in large number.

3.1.1. Callus Induction and Reproduction in MM.106 Cultivar on BAP, IBA Treatment

We investigated embryonic and non-embryonic callus through cultivating apical and lateral buds on different treatments with MS base culture and BAP (0-2.5 mg/l) and IBA (0- 5 mg/l) hormones indicated in table 1. The largest size for callus was obtained as 10.4mm in T23 treatment with BAP (2.5 mg/l) and IBA (4 mg/l) in third sub-culture and embryonic calluses were formed in T_{11}, T_{13} and T_{15} treatments. Callus induction began from 5th week. Calluses were brittle and granular with white, cream, green and light brown colors.

Table 1. *MS treatment with NAA, Kin hormones (Daigny et al., 1998).*

Kin (mg/l) \ NAA (mg/l)	0	1	2	3
0.5	K1	K2	K3	K4
1	K5	K6	K7	K8

By comparing callus induction to leaf explants and lateral and apical buds, we found that leaf explants respond better to callus induction. After formation of embryonic calluses in induction environments, they were transferred to their original environment after two weeks for reproduction and embryogenesis.

3.1.2. Callus Induction and Reproduction in MM.106 Cultiva on NAA, Kin Treatment

The explants of apical and lateral buds and the leaf were cultivated in MS treatment with Kin and NAA hormones (table 2). Induction and production of embryonic callus was investigated. Among 8 treatments, K_2 (NAA 1 mg/l, Kin 0.5 mg/l) and K_8 (NAA 3 mg/l, Kin 1 mg/l) yielded the highest amount of callus (5.3 mm) and in statistical analyses the data according to variance analysis were not significant in 0.05 probability level.

Table 2. *MS treatment with BAP, IBA hormons (Jacoboni, satandardi 1982).*

BAP (mg/l) \ IBA (mg/l)	0	1	2	3	4	5
0.5	T_1	T_2	T_3	T_4	T_5	T_6
1	T_7	T_8	T_9	T_{10}	T_{11}	T_{12}
2	T_{13}	T_{14}	T_{15}	T_{16}	T_{17}	T_{18}
2.5	T_{19}	T_{20}	T_{21}	T_{22}	T_{23}	T_{24}

3.2. Reproduction of Embryonic Callus

We sub-cultured the formed calluses on primary treatments and after 4 weeks we investigated the calluses. In cultivar MM. 106 on treatments of T_2 (IBA 1 mg/l, BAP 0.5 mg/l), T_{11} (IBA 4 mg/l, BAP 1 mg/l), T_{13} (IBA 0, BAP 2 mg/l), T_{15} (IBA 2 mg/l, BAP 2 mg/l), T_{23} (IBA 4 mg/l, BAP 2.5 mg/l), T_{24}(IBA 5 mg/l, BAP 2.5 mg/l) and K_2 (NAA 1 mg/l, Kin 0.5 mg/l) treatments, embryogenesis was achieved and percent of embryogenesis was 8-10%.

The highest percent of embryogenesis related to T_{11} treatment (IBA 4 mg/l, BAP 1 mg/l) and T_{23} (IBA 4 mg/l, BAP 2.5 mg/l). The embryos were in corpuscular stage. Then third sub-culture was performed and all calluses and embryos were delivered to their corresponding treatments. The embryos remained in corpuscular stage and they did not show reproductive ability and developmental process.

3.3. Callus Induction and Reproduction in M.26

After callogenesis, in order to form embryos, each callus was sub-cultured on its primary treatment for one month. In fourth week, the calluses were investigated and M. 26 cultivar did not form any embryo.

3.4. Preparation of Artificial Seed

We prepared synthetic seed as was mentioned in materials and methods then We investigated regeneration of artificial seed each week for 2 months. No regeneration was seen.

4. Discussion and Conclusion

4.1. Discussion and Conclusion Regarding Induction and Production of Embryonic Callus of Two Cultivars of Apple, Malling Base

The differences between embryonic and non- embryonic calluses are based on morphology and physiology, metabolism and patterns of gene expression that is consistent to [7] Frey's reports (1992)

In this study, 32 treatments were used for callus induction in two cultivars, M. 26 and MM. 106 and it seems that cultivation of apple tissue depends on genotype, such that the results relating to mentioned cultivars were different.

In this research, all callus induction treatments performed callogenesis for MM. 106 cultivar, but just two treatments were suitable for callogenesis of M. 26 cultivar. The results indicated that the type of treatment and cultivar impact induction percent, color, state and tissue of callus. In some

cases, explants of a single cultivar yielded different calluses in terms of color and tissue on a treatment. These differences may be attributed to the amount and type of reserves of explants hormones and or the differences among explant segments.

Figure 1(a). *Embryogenic callus.*

Figure 1(b). *Non embryo genic callus in T_{11} treatment.*

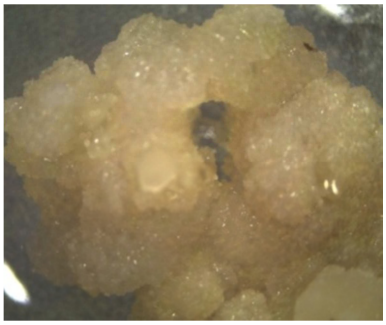

Figure 2(a). *Embryo genic callus.*

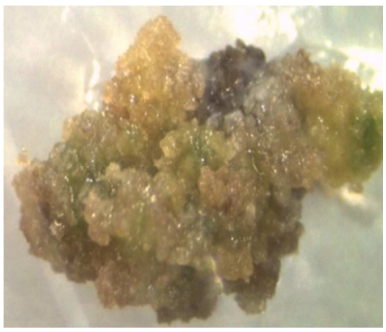

Figure 2(b). *Non embryo genic callus in T_{15} treatment.*

Among used treatments, those containing IBA and BAP showed better cllogenesis in terms of value and speed of callogenesis compared to treatments containing NAA and Kin. But the value and speed of callogenesis in treatments containing NAA and D-2, 4 are similar to that of IBA and BAP contained treatments. [4] Daigny et al. (1990) used cotyledon or immature apple seedling *Malus X domestica Borkh* cv. *Golster* G9 and found that various factors are effective in callogenesis including NAA, BAP, Kin or TDZ hormones that was consistent to the results of present study.

The calluses of MM. 106 cultivar were larger and better than that of M. 26. This difference relates to different reactions of tissue to callogenesis environment that depends on internal hormones level. In this study, the optimum place for production of embryonic callus is 5-8 treatments and application of NAA and D- 2, 4 hormones in combination had no effect on embryogenesis. After three weeks, the calluses reduced in growth and their color and state changed which was probably due to reduction of macro/micro salts, reduction of vitamins and hormones and phenolic and toxic materials increase.

Embryogenesis is a critical stage in the life of higher plants. Embryogenesis was performed in low percent in present study and embryos died in corpuscular stage, so that the embryos could not grow.

Somatic embryogenesis is also dependent to genotype, such that concerning two cultivars of M. 26 no embryogenesis was achieved, while for MM. 106, a few embryos were observed. [16] Paul et al. (1994) used zygotic cotyledon and embryo for embryonic callus formation and they reported only 30% embryogenesis.

[9] James et al. (1984) applied different parts of apple bases M9, M25, M26 and M27 for embryogenesis in MS treatment together with NAA and BAP hormones.

4.2. Discussion and Conclusion Concerning Artificial Seed Production in Two Cultivars of Apple, Malling Base

Successful application of artificial bud as graft involves an efficient and capable of vegetation system to achieve a strong plant. It was Murashige [15] (1979) who for the first time talked about artificial buds production, but this task faced delay for many years. Producing artificial seed from vegetative parts of plants has many advantages including: rapid propagation of same clones, maintenance of germplasm and plant breeding which is beneficial in plants that are unable to produce natural seeds or suffer genetic non-uniformity and where there are storage and transportation problems [19] Ravi et al. (2012). Using encapsulation of somatic embryos in some plants such as tobacco, coffee, soya bean, cotton and beans, [23] Fujii et al (1989) obtained artificial seeds, but in some plants such as carrot and alfalfa they succeeded.

Regarding the results, we found that embryogenesis was very weak in these two cultivars and the embryos died in corpuscular stage, so artificial seeds were obtained from apical and lateral buds of these cultivars. [12] Mehra and Sachdeva (1984) reported artificial seeds production in plants lacking embryos and they used M26 maling base; in our research, we put vegetative buds (apical and lateral buds) of apple, malling bases M. 26 and MM. 106 in sodium alginate capsule and we developed artificial seed capsule around the buds. [26] Tsvetkov et al. (2005) put apical buds and nodes

of oak stem in capsule. Sodium alginate is a matter which takes gel form with water and it is non- toxic and safe and it is easily takes a solid form and it is available [2] Barbotin et al. (1993) its gel state makes this matter to produce covalence band with pH changes [8] Edward-Levy(1996).

According to [21] Samrah et al.'s (2010) reports a part of plant, such as bud of embryo must be encapsulated for direct planting of seeds in farm or greenhouse. The material used for encapsulation must be harmless and protect the plant from pathogens, non-suitable conditions, and mechanical and transportation damages and at the same time provide required nutrients for its growth to become a seedling and also provide gaseous exchanges and respiration. [13] Micheli et al (2002) reported artificial seed preparation from M. 26 (*Maluspumila Mill*); sodium alginate capsule in artificial seed prevents the plant from dehydration, mechanical damages during transportation and nutrient reduction and artificial seeds of M. 26 and MM. 106 cultivars were stored for 2 months under sodium alginate capsule that is consistent to mentioned results.

Malling bases have very tiny seeds and immature embryos, so they are suitable options for artificial seed preparation; by encapsulating the vegetative parts of M. 26 and MM. 106 cultivars, propagules were easily stored. [18] Besides Rai et al. (2009) reported encapsulation technology for seedless fruits or heterozygote seeds with low endosperm and germination rate. [3] Brischia et al. (2002) produced artificial seeds in malling base of M. 26 cultivar and added that encapsulating other vegetative parts (propagule) may reduce transportation costs in production. Artificial seeds of M. 26 and MM. 106 cultivars were stored in 4°C for 60 days in refrigerator; the seeds showed regenerative capability and we investigated their germination rate. No regeneration was observed in those two cultivars. [17] Rady et al (2004) stored artificial seeds obtained from apical bud of Gypsophila plant in vitro in 4°C and they observed the irregeneration after 30-90days.

4.3. Conclusion

Some of advantages of artificial seeds are: prevention from dehydration and glassification propagules that could be stored under cryopreservation conditions [5] Faisal et al. (2006) besides, [25] Standardi et al. (2013) reported encapsulation of plants as a new tool for transportation in nursery greenhouses.

Embryogenesis was performed in low percent in present study and embryos died in corpuscular stage, so that the embryos could not grow. It seems that cultivation of apple tissue depends on genotype, such that the results relating to mentioned cultivars were different. Regarding the results, we found that embryogenesis was very weak in these two cultivars and the embryos died in corpuscular stage, no artificial seeds were obtained from apical and lateral buds of these cultivars the aim of this research is a useful protocol about somatic embryogenesis in two cultivars of apple. since somatic embryogenesis is a useful method for genetic changes then more studies with other concentration of hormones (other types of auxin and cytokines) is recommended.

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