

Article

Scanning electron microscopic and morphological studies on in-vitro derived callus of low-chill peach (*Prunus persica* L. Batsch)

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Abstract: In this investigation the effect of collection seasons of explants (winter, spring and summer), type of explants (leaf disc and intermodal segments) and length of explants (0.5, 1.0 and 1.5 cm) for callusing in low-chill peach were standardized. The maximum callus induction (97.78%) in the low-chill peach was obtained from the intermodal segments of 0.5 cm in length used as an explant collected during spring season. The structural changes on the surface of the callus (5–7 weeks old yellowish green compact callus) during the progress of somatic embryogenesis of low-chill peach from the both intermodal segment as well as leaf disc derived callus were also examined with the use of scanning electron microscope (SEM). The SEM studies indicated that callus derived from internodal segment explant had the highest frequency of somatic embryos than callus from leaf discs. The SEM investigation, also demonstrated the sequential events/steps leading to low-chill peach somatic embryogenesis which was originating from somatic embryo mother cells through one unicellular pathway. Two types of calli were morphologically distinguished in both leaf disc and intermodal segment generated callus and these were the compact, well organized yellowish green embryogenic callus, containing large number of small, rich cytoplasmic, starch containing meristematic cells and soft and unorganized non-embryogenic callus containing sparsely cytoplasmic, vacuolated, and large cells devoid of metabolic reserves. The present SEM studies clearly demonstrated that somatic cells from peach explants generated callus could develop into fully differentiated somatic embryos through the characteristic embryological patterns of differentiation.

Keywords: callus; scanning electron microscopy; MS basal medium; embryogenic; non-embryogenic

1. Introduction

Peach [*Prunus persica* (L.) Batsch] is one of the most important temperate stone fruit crops of the world. It belongs to the family Rosaceae and subfamily Prunoideae. Being a tree species, regeneration of peach through plant tissue culture technique is a challenging job and in-vitro callus establishment is an important intermediate step in peach biotechnology [1]. Callus is an undifferentiated and/or differentiated, actively-dividing, non-organized mass of cells often developing either from injury/wounding or cut surfaces in tissue culture media supplemented with plant growth regulators. A tiny live plant part either mature or immature in nature can be used to regenerate callus. The regenerated callus under tissue culture exhibited several kinds of morphological

variation ranges from white or coloured, soft (watery) or hard, friable (easy to separate into cells) or compact. Such kind variations in in-vitro derived callus differs between plant to plant, species to species and varieties to varieties. The initiation, induction and growth rate of callus within a plant species is also depending upon various factors such as, the physiological status and plant part used as explant. Although, the callus remains unorganized, with increasing growth, some kinds of diversified specialized cells may be associated with centres of the morphogenesis of roots, shoots and embryos [2]. Callus tissues can be formed by placing small pieces of explants on a growth medium under sterile condition in-vitro. The nature and concentration of used plant growth regulators in tissue culture media have also played a very significant role in callus induction process [3]. Auxin and cytokinin are the two most widely known plant growth regulators used in plant tissue culture mediums. The combination and concentration of both these plant growth regulators ultimately determines the type of culture established. Scientific evidence revealed that callus can be generated artificially under in-vitro conditions and the balance between two plant hormones i.e., auxin and cytokinin, determines the state of differentiation and dedifferentiation of callus. Further, the number of in-vitro regeneration cycles undergone by the donor explant determines the size of induced the callus [4]. Different types of calli are used to serve specific purposes. Suppose, for embryogenesis white and globular calli are used [5], while green and nodular calli are suitable for organogenesis [6–8]. This technology has been widely used in both basic research and industrial applications [9].

In-vitro culture of plant cells, tissues or organs in an artificial growing medium containing selective agents offers the opportunity to select and regenerate plants with desirable characteristics. Scanning electron microscopy (SEM) research clearly reveals the process of somatic embryogenesis in callus tissues. Somatic embryogenesis initiated from compact, granular sectors of a callus and often-observed somatic embryos suggested that somatic embryogenesis is a major pathway for callus regeneration [10]. Histological studies showed that in the nodular embryogenic calluses, the outlying parenchymal cells exhibited cellular characteristics of high mitotic activity. Differentiation of tracheal elements exists in embryogenic calluses connecting or jointing the callus to the explant. The evaluated cytokinin/auxin combination and interaction influences the development of embryogenic calluses and globular structures. Usually, a high concentration of the auxin in the culture media is necessary for the induction of nodular embryonic structures in peach palm during somatic embryogenesis [11]. Histological studies also showed that starch accumulation leads to the development of somatic embryos, and these cells had high nucleus/cytoplasm ratios and high mitotic activities.

Somatic embryogenesis is the developmental process where a plant somatic cell can dedifferentiate to totipotent embryonic stem cell and having the ability to give rise an embryo under appropriate conditions. Such newly generated embryos can further develop into whole plants. In woody fruit trees, somatic embryogenesis plays a critical role in clonal propagation and also a powerful biotechnological tool for synthetic seed production, germplasm conservation, and cryopreservation. A key event in somatic embryogenesis is the transition of cell fate from a somatic cell to embryo cell. Somatic embryogenesis has already been widely applied by researchers for a number of fruit crop species and propagation through plant tissue culture in adult woody fruit trees till

remains problematic. In this article, we are basically focusing on the process of in-vitro callus induction and understanding the mechanisms of somatic embryogenesis and its practical applications in a woody fruit tree i.e., low-chill peach by scanning electron microscopic study. Additionally, we also propose a strategy to improve the process of somatic embryogenesis using callus culture techniques for low-chill peaches.

2. Materials and methods

2.1. Explant collection

The explants were collected from 5–8 cm long healthy and newly flushing current season twigs of a 6 years old low-chill peach cv. Early Grande tree orchard located at Horticultural Research Centre (243.84 meters above MSL and at 29° N latitude and 79.3° E longitudes), G.B. Pant University of Agriculture and Technology, Pantnagar during winter, spring and summer. The region experiencing humid subtropical climate popularly known as ‘Tarai’ tract. Two types of explants viz., leaves and internodal segment explants were cut from the collected current season twigs with the help of a sharp Ni coated blade and kept immediately in 500 mL jam bottle containing 200 mL water during morning and brought to the tissue culture laboratory for further processing.

2.2. Washing, sterilization and processing of explant

The collected explant materials were dipped into 100 mL of distilled water containing 0.5 mL Teepol (liquid detergent) and 0.1 g Bavistin for 5 min to remove the darts followed by washing under running tap water for about half an hour to remove the stucked detergent. The materials were then rinsed with distilled water [12,13]. After treatment with Bavistin for 30 min and washing thoroughly in running tap water, the explants were brought to the laminar air flow cabinet for surface sterilization treatments. Three surface sterilants viz., 70.0% ethanol, 0.1% HgCl₂ and 1% NaCl were used for different durations. The explants were further rinsed with sterile distilled water (3–4 times) and dried in pre-sterilized tissue paper sheets. The dried explants both leaf discs and internodal segments were cut into different sizes (0.5 cm, 1.0 cm and 1.5 cm) using Ni coated blade.

2.3. Culture establishment for callus induction

All explants, namely leaf discs with mid rib (1 cm) and internodal segments (1 cm) were used for culture establishment for callus induction. Explants were placed horizontally in callus induction media consisting of MS medium supplemented with BAP (0.5 mg L⁻¹) and 2,4-D (0.5 mg L⁻¹). The cultures were then incubated at 22 ± 2 °C in continuously dark.

2.4. Callus induction (%) and callus morphology

The callus induction percentage were recorded 5 weeks after the establishment of the explants in the media and calculated by using formula given below.

$$\text{Callus induction \%} = \frac{\text{Number of explants forming callus}}{\text{Total number of explants culture}} \times 100$$

The colour of callus, callus type and callus growth were recorded on a visual scale observation 7 weeks after incubation.

2.5. Scanning electron microscopic study

Some characters related to cell morphology were observed in generating as well as non-generating cells so as to find the types of cell masses undergoing regeneration and also find out the differences in callus development. The callus used for recording fresh weight of callus also used for SEM study. Firstly, the calluses were cut in 1–2 mm sample size with the help of sharp surgical blade. After that the cut callus pieces fixed in 2.5% gluteraldehyde solution for 2 and half hours at 4 °C. Then 3 times washing with phosphate buffer (PBS) pH 7.2–7.4 each for 10 min. Then the samples were dehydrated with sequentially with a series of alcohol 30%, 50%, 70% and 90% at 4 °C temperature and finally 100% at room temperature. The callus samples were dried to their critical drier point (CDP) and were coated with gold plating [Model No. JFC-1600, Japan Electronic Optical Limited (JEOL)]. The specimens were examined and photographed in a Scanning electron microscope (EM-6610) at different magnification.

2.6. Statistical analysis

The per cent data were transformed subjected to angular transformation before statistical analysis. The results are presented with the help of tables, graphs and photographs. The significance of variance among the treatments were observed by applying ‘*F*’ test and critical difference (CD) at 5 per cent level of significance was calculated to compare the treatment means for all the characters. The observations were recorded for growth pattern, type, colour and percentage of callus obtained from each type of explant after culturing and the experiment was repeated thrice and statistically analyzed under factorial completely randomized design.

3. Results

In the course of annual cycle, the peach tree undergoes its internal equilibrium evolve: during the spring the organs grow and analyses exhibit the presence of growth regulators such as auxins, gibberellins and cytokinins. During the summer, the flow of substances diminishes and then during the autumn and winter, the inhibitors appear. Thus, cultured tissues when put on the media, show different responses. Therefore, during the course of investigation the effect of explants collection season, type and length of explants for callusing in peach were standardized with following measurements.

3.1. Callus induction

Callus induction was observed at the cut edge of explants that showed continuous increase in size leading to callus formation. Significantly higher callus induction was observed in the explants collected during spring season (94.44%) followed by summer (80.37%) and winter season (8.15%) (**Figure 1A**).

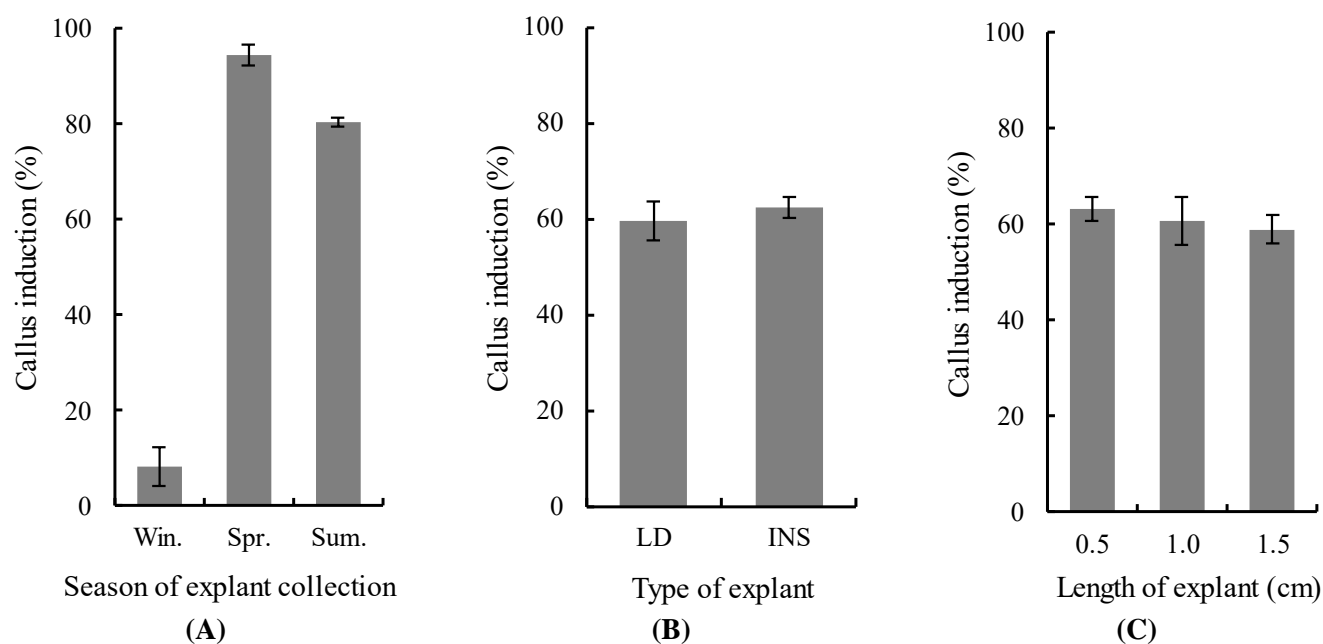


Figure 1. The main effect. **(A)** season of explant collection; **(B)** type of explant; **(C)** length of explants.

Win.: Winter, Spr.: Spring, Sum.: Summer; LD: Leaf disc, INS: Internodal segments. The vertical bar denotes standard error ($n = 3$) and the same letters are not significant at $P \leq 0.05$.

However, the effect of type of explants on callus induction percentage was found to be significant (**Figure 1B**). The highest callus induction (62.47%) was recorded in internodal segment as compared to the leaf disc explant (59.51%).

The length of the explants also significantly affected the callus induction and found to be as one of the most critical factors of callusing in low-chill peach (**Figure 1C**). With the increase in length of explants, there was significant decrease in callus induction. The maximum callus induction (63.33%) was observed in explants measuring 0.5 cm length followed by 1.0 cm in length (60.74%). The explants measuring 1.5 cm in length had resulted minimum callus induction (58.89%). However, each length of explant was significantly different over other counterparts.

The effect of interaction (season of collection of explants \times type of explants) on per cent callus induction was found to be non-significant (**Figure 2A**). However, the least per cent callus induction (6.67%) was recorded in the leaf discs collected during winter season and that was closely followed from the internodal segments also collected in the winter season (9.63%). The callus induction percentage was the highest (95.55%) from the internodal segment explants collected during spring season followed by leaf disc explants collected in spring (93.33%).

The effect of interaction (season of collection of explants \times size of explants) on per cent callus induction was also found to be non-significant (**Figure 2B**). The callus induction (6.67%) was recorded least when explants size 1.5 cm taken during winter season. However, the callus induction percentage (7.78%) was also nearly similar to minimum when size of explants is 1 cm and used during the winter season. The highest per cent callus induction (96.67%) was noted when explants size is 0.5 cm and was collected during spring closely followed by (94.44%) when explants size is 1.5 cm and was collected during spring in both type of explants. However, each treatment combination was different over other counterparts.

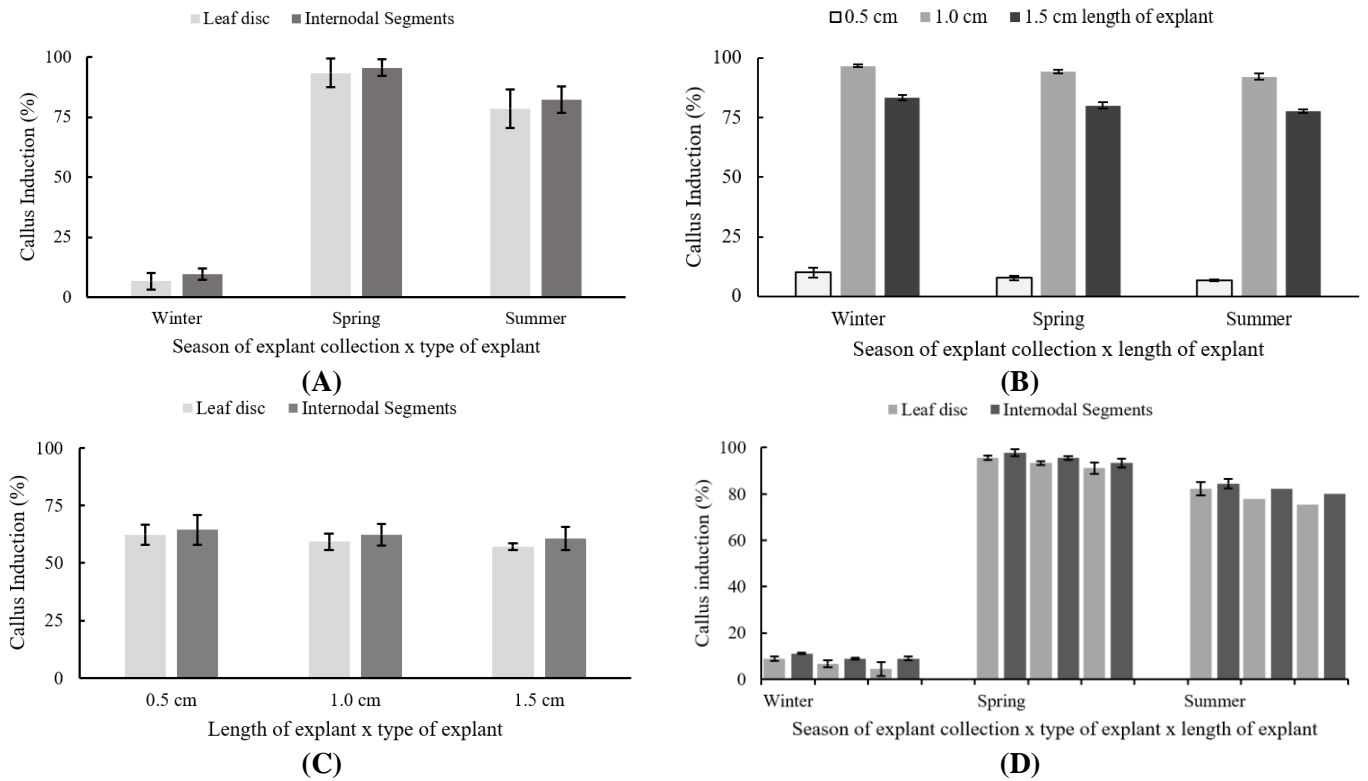


Figure 2. The interaction effect. (A) season of explant collection \times type of explant; (B) season of explant collection \times length of explant; (C) length of explant \times type of explant; (D) season of explant collection \times type of explant \times length of explant.

The vertical bar denotes standard error ($n = 3$) and the same letters are not significant at $P \leq 0.05$.

The effect of interaction (type of explant \times length of explant) on per cent callus induction was also found to be non-significant (**Figure 2C**). The least per cent callus induction (57.04%) was recorded in the 1.5 cm length leaf disc of explants followed by (59.26%) in leaf disc of 1.0 cm size explants length. The maximum per cent callus induction (64.44%) was noted the highest in 0.5 cm length internodal segments followed by (62.22%) 0.5 cm in leaf disc explants and 1.0 cm length of internodal segment explants.

The effect of interaction (season of collection of explants \times type of explants \times size of explants) on per cent callus induction was also found to be non-significant (**Figure 2D**). However, the callus induction percentage in low-chill peach was noted the highest (97.78%) when 0.5 cm length internodal segments were collected during spring season which was closely followed by the explants collected during the same season from 0.5 cm length leaf discs explants (95.55%). The least per cent callus induction (4.45%) was recorded when 1.5 cm leaf disc were used as explants during winter season followed by 1.0 cm leaf disc explants collected during winter season (6.67%).

3.2. Callus morphology

Observation on callus morphology indicate that the leaf disc explants collected during winter season formed white, watery type callus with poor callus growth while no callus formation was noticed in internodal segments collected during winter season. However, the leaf disc and intermodal segments collected during spring formed

excellent friable yellowish green callus and compact green coloured callus, respectively. During summer season the calli produced from leaf disc explants were found to be friable type with brownish colour. However, the internodal segments collected during summer season produced whitish green semi-compact types callus with fair growth habit. Thus, this study confirms that season of collection of explants, type of explants and their length had significant effects on callus morphology.

In present investigation significant influence of explants collection season on callus morphology was observed and the variation on callus morphology across the collection season with respect to type of explants might be due to the variation in chlorophyll pigmentation within the explants in different season.

3.3. SEM observation

Cell divisions in plants take place in the meristems. Parenchyma (non-dividing) and the cells in meristems, vascular cambial tissues and embryonic tissues are at early (initial) stages of development and exist in an ‘undetermined’ state which can rapidly proliferate to produce calli. Callus is an amorphous mass of unorganized thin-walled parenchyma cells induced and formed from the cut surface of the explant tissue. Callus formation took place 2–3 weeks after the establishment of the explants in MS media supplemented with auxin. However, the complete formation of callus over the surface of the explants ended 5–6 weeks later. At the beginning of culture, the calli were greenish white (leaf disc explant generated callus) or yellowish green (internodal segment explant generated callus) coloured (**Table 1, Figures 3A and 4A**). In the following weeks the calli were friable and compact largely proliferated and turned greenish brown respectively in leaf disc and internodal segment generated callus. Better callus formation was observed from the internodal segment explants cultured on MS medium supplemented with BAP and 2,4-D. Calli and the localized groups of glossy cell aggregates on their upper surfaces, treated with 2,4-D plus BAP invariably gave rise to more glossy, translucent, globular and slightly elongated protuberances. Here morphogenic response clearly appeared only in certain sectors of the compact callus. Scanning electron microscopic (SEM) analysis was used to verify the morphological observation of the compact shape structures induced in the internodal segment explants of peach callus. SEM studies of 54 days old greenish white friable (leaf disc explant generated callus) or yellowish green compact (internodal segment explant generated callus) callus revealed (**Table 1, Figures 3B and 4B**), several surface structural changes during somatic embryogenesis (**Figures 3C and 4C**).

Table 1. Effect of season of explant collection, type of explant and length of explants on callus morphology in peach.

		Callus morphology									
		Season (A)									
		Size of explants (C)	Winter (S ₁)			Spring (S ₂)			Summer (S ₃)		
Type of explants (B)			Callus colour	Callus type	Callus growth	Callus colour	Callus type	Callus growth	Callus colour	Callus type	Callus growth
(B)	Leaf disc (E _i)	0.5 cm	W	W	+	GW	F	++++	GB	F	+++
		1.0 cm	W	W	+	GW	F	++++	GB	F	+++
		1.5 cm	W	W	+	GW	F	++++	GB	F	+++

Table 1. (Continued).

		Callus morphology									
		Size of explants (C)	Season (A)								
			Winter (S ₁)			Spring (S ₂)			Summer (S ₃)		
Type of explants (B)		Callus colour	Callus type	Callus growth	Callus colour	Callus type	Callus growth	Callus colour	Callus type	Callus growth	
(B)	Internodal segment (E ₂)	0.5 cm	-	-	-	YG	C	++++	LG	SC	++++
		1.0 cm	-	-	-	YG	C	++++	LG	SC	++++
		1.5 cm	-	-	-	YG	C	++++	LG	SC	+++

Callus colour: W = White, WG = Greenish white, LG = Light green, YG = Yellowish green, GB = Greenish brown. Callus type: F = Friable, SC = Semi-compact, C = Compact, W = Watery. Callus growth: += Poor, ++ = Average, +++ = Good, ++++ = Excellent.

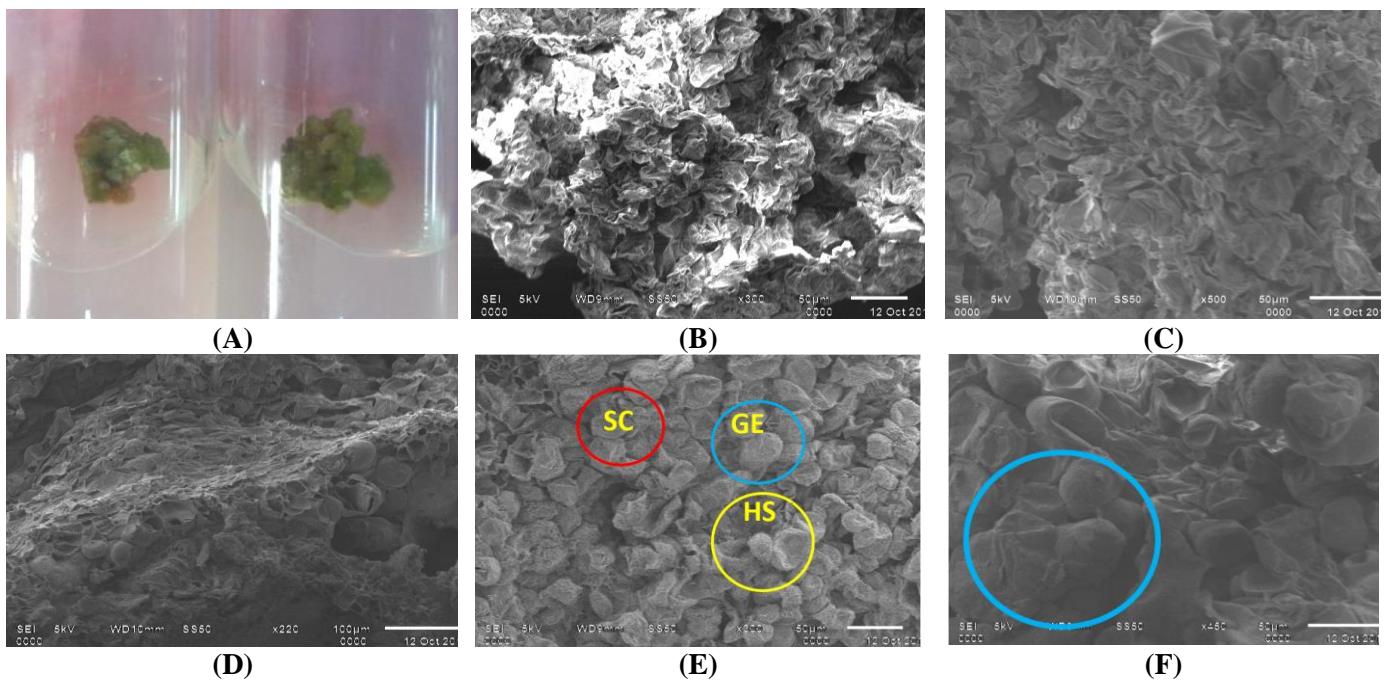
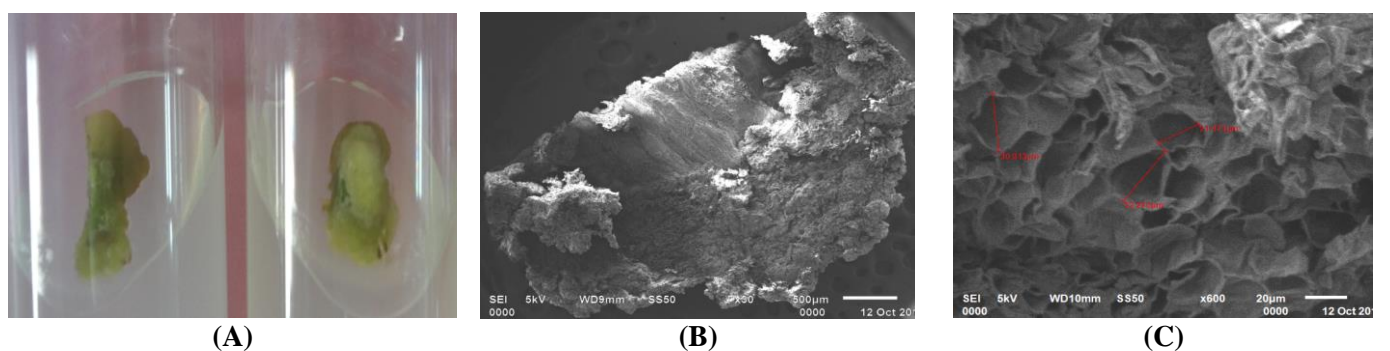


Figure 3. (A) greenish white friable callus from leaf disc explant; (B) surface image of leaf disc explants generated friable callus in scanning electron microscope; (C) non-embryogenic callus from leaf disc explants showing small dense unorganized structure of cells; (D) developmental changes from non-embryogenic to embryogenic callus of leaf disc showing different shaped unorganized structure of cells; (E) embryogenic callus from leaf disc with different shaped organized cell growth showing developmental changes; (F) leaf disc explant generated embryogenic callus showing individual cell size in scanning electron microscope.

SC = Spherical cell from embryogenic callus; GE = Globular embryos emerging from embryogenic callus; HS = Heart shaped embryo emerging from embryogenic callus.



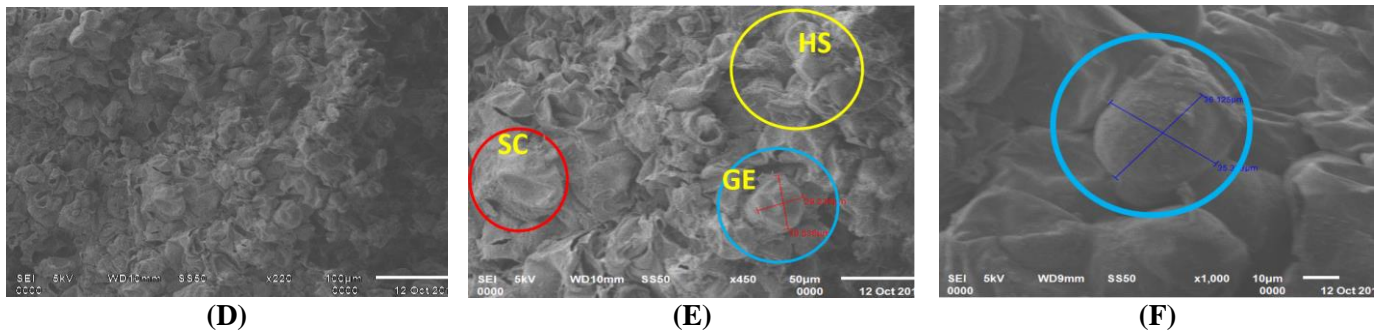


Figure 4. (A) yellowish green compact callus from intermodal segment explant; (B) surface image of intermodal segment explants generated compact callus in scanning electron microscope; (C) non-embryogenic callus from intermodal segment explants showing small dense unorganized structure of cells; (D) developmental changes from non-embryogenic to embryogenic callus of intermodal segment showing different shaped unorganized structure of cells; (E) embryogenic callus from intermodal segment with different shaped organized cell growth showing developmental changes; (F) Internodal segment explant generated embryogenic callus showing individual cell size in scanning electron microscope.

SC = Spherical cell from embryogenic callus; GE = Globular embryos emerging from embryogenic callus; HS = Heart shaped embryo emerging from embryogenic callus.

Each embryonic structure had its own characteristic epidermal layer. SEM image (Figures 3D and 4D) revealed that the embryos were globular with smooth surface. Under SEM analysis, it was observed that the cell masses developed under the influence of MS medium supplemented with BAP and 2,4-D had a round shape resembling the compact cell mass. The image presented indicates the individuality of globular structure (Gs).

The section of the initial explant showed highly vacuolated central core of large parenchymatous cells, surrounded by outer mantle of vascular bundles (Figures 3E and 4E). The stem primordia appeared as a round part that stands out from the surface of meristematic cells. After 2–3 weeks on the medium, a section of explant segments revealed actively dividing richly cytoplasmic cells at the cut end of intermodal segment. The meristematic cells at the periphery were small and compact, with densely formed cells. After 3–5 weeks of culturing, these cells differentiated into medium sized structures showing non-embryogenic callus cells (Figures 3D and 4D).

These non-embryogenic callus cells were easily distinguished from the proactive epidermal layer due to their greater size and dense structure. After 5 weeks of culture, these highly dividing non-embryogenic cells occupied several layers immediately after the epidermis, followed by small, large and medium sized cells with prominent nucleus, representing various stages of differentiation (Figures 3E and 2E). Discrete groups of cells on the callus surface formed directly from internal segmenting divisions in many of these cells. Each and every group were distinct and separated from each other by thick cell wall. A rapid and localized proliferation was clearly observed in activity meristematic zone, producing a lobed callus showing the absence of vascular connection with the parent tissue and the development of vascular initials with the lobed callus which revealed features of early embryogenic callus development. This lobed structure termed as embryogenic tissue contained several layers of embryogenic cells (Figures 3E and 4E).

Continued divisions and organization in some of these discrete groups formed

rounded structures pro-embryoids with distinct epidermis. Further differentiation of these structures led to the formation of globular embryoids, which appeared on the surface of the callus after breaking through the epidermis. These globular structures developed into somatic embryos on the surface of the callus when maintained by subculturing for more than 6 weeks (**Figures 3F** and **4F**).

A general surface morphology of a cluster of compact non-embryogenic callus undergoing the differentiation in the callus surface was rough. The callus was comprised of two different plant parts, one was a compact yellowish green callus from intermodal segment (**Table 1, Figure 4A**) and the other callus was friable whitish green from leaf disc (**Table 1, Figure 3A**). The friable callus is composed of cells with a spherical shape and the compact callus consists of round to elongated cells and a membranous layer covering them. Sometimes the spherical cells are also covered with a membranous layer. The arrangement of both spherical isodiametric cells and an elongated tubular cell is clearly illustrated in the figures. The large nodules are composed of spherical cells as shown in the figure. Under high magnification, the arrangement of isodiametric spherical cells on the surface of a cluster of a friable embryogenic callus can be easily observed. Resulted spherical structure is growing putative pro-embryogenic stage derived from a single cell of about 15 μm diameter.

One of the Figures (**Figure 4E**) illustrates that single cells are of about 35.393 μm to 36.125 μm in diameter and are interconnected and that a membranous layer is slightly covering both cell surfaces. In the advanced stages of differentiation, spherical cells are sprouting from the basal region of a developed embryo. Resulted differentiation pattern clearly illustrates secondary embryo formation from primary somatic embryos. Early globular embryos with well-developed suspensors have been observed. **Figure 4E** also shows that a typical globular embryo stage is emerging from the callus surface still interconnected by the suspensor. An early heart shaped embryo with a slight depression on the tip and one slight elongated form of one globular embryo has been observed (**Figures 3E** and **4E**). In **Figures 3E** and **4E** clearly shows the state that typical growing heart shaped and globular shaped embryos are emerging from a compact embryogenic callus located in the vicinity of well-developed embryos. This developmental pattern illustrates embryo formation at the border between developed embryos also in friable embryogenic callus (**Figures 3F** and **4F**).

In present study, two types of calli were morphologically distinguishable in both leaf disc and intermodal segment generated callus. The compact, well organized and yellowish green embryogenic callus containing large number of small, rich cytoplasmic, starch containing meristematic cells and soft, unorganized non-embryogenic callus containing sparsely cytoplasmic, vacuolated, and large cells devoid of any prominent metabolic reserves. In peach non-embryogenic callus had enlarged cells that were loosely arranged on its surface. Somatic embryogenesis initiated from compact, granular structures on the callus surface. In some isolated sectors of non-embryogenic callus, there was the formation of masses of embryogenic callus (**Figures 3E** and **4E**). These sectors of embryogenic callus possibly originated from a few original groups of embryogenic competent cells scattered amongst non-embryogenic cells. Conversion of non-embryogenic cells into embryogenic cells was never been convincingly demonstrated (**Figures 3E** and **4E**). Embryogenic callus proliferated more slowly, especially during initial phases of growth than non-

embryogenic callus. Selective transfer of embryogenic callus was done at each subculture in order to maintain its embryogenic nature. A friable and fast-growing embryogenic callus type was produced either directly from leaf disc explants or after selection from white friable embryogenic callus in peach. The embryogenic cells were found to be small and highly cytoplasmic with rapid cell division occurring in a determined locus of the cell cluster formed globular embryos. The high frequency of somatic embryos was found in callus tissues which derived from internodal segment explants and low frequency of somatic embryogenesis was found on the leaf edges associated with very small callus formation and also from the callus tissues derived from young leaf disc explants.

Based on the Scan Electronic Microscopic (SEM) observation on the somatic embryogenesis in Peach tissue cultured various stages of embryo differentiation in this system can be seen. There are seven distinct stages recognized in the somatic embryogenesis process of Peach callus tissue culture in this present work cited.

- 1) Somatic single cell/embryo mother cell
- 2) Early globular embryo
- 3) Globular embryo
- 4) Elongated embryo
- 5) Early heart embryo
- 6) Heart embryo
- 7) Torpedo embryo

Scanning electron microscopy research clearly revealed somatic embryogenesis in peach callus under in-vitro. SEM observations showed that in the nodular embryogenic calluses, the outlying parenchymal cells exhibit cellular characteristics of high mitotic activity. Differentiation of tracheal elements exists in embryogenic calluses connecting or jointing the callus to the explant. The evaluated cytokinin/auxin combination and interaction influences the development of embryogenic calluses and globular structures. Combination of BAP with the auxins (2,4-D) is not necessary in the pre-treatment of the explants for the formation of primary calluses during the somatic embryogenesis in peach. SEM analysis was used to verify the morphological observation of the globular shape structures induced from the callus generated by internodal segment explant. Under SEM analysis, it was observed that the cell masses developed under the influence of BAP and 2,4-D had a rounded shape resembling the pro-embryo structure (**Figures 3E** and **2E**). The image presented in this figure indicates the individuality of globular structure (Gs).

4. Discussion

Scientific literatures suggested that cell division takes place in the meristematic tissues of the plant. Parenchyma (non-dividing) and the cells in meristems, vascular cambial tissues and embryonic tissues are at early (initial) stages of development and exist in an 'undetermined' state which can rapidly proliferate to produce calli/callus. Plant species with continuous growth habit throughout the year are usually easier to form callus than species characterized by episodic and flushing growth pattern [14]. The season of the year, donor conditions of the plant, the age and physiological state of the parent plant are responsible for the successful callus culture. A serious problem

which generally faced when culturing winter season explant of the Rosaceous fruit tree species, is the oxidation of phenolic substances that generally leached out from the cut surface of the explant which turns the medium to dark, brown colour and is toxic to the tissue [15].

The results obtained in the above experiment are in conformity with those of successfully initiated callus from 5 mm stem internodes of peach, plum, cherry and apricot [16]. Chen and Jonard [17] recorded cent percent induction of callus using stems as explant in apricot. It is also reported that peach (cv. Sylvestris and Red Haven) shoot-tips collected during the June were able to produce callus. Schiavone and Wisniewski [18] successfully induced callus from peach internodal segments of stems collected during the month of January. Thus, peach has specific requirements to produce callus. Among the all three seasons, spring season was found to be the best season with regard to callusing percentage, degree of cell division, minimum days taken to callus initiation and formation, minimum fungal and bacterial contamination in both types of explants.

The selection of appropriate explant is the most critical factor of successful callus induction. Since, the texture and type of derived callus is mainly depended upon explant type. Further the combination and concentration of used growth regulators in the tissue culture media play a significant role in callus induction process. Generally, the callus tissue from different plant species may be different in structure and growth habit viz. white or coloured, soft (watery) or hard, friable (easy to separate into cells) or compact. Although, the callus remains unorganized, with increasing growth, some kinds of specialized cells may be associated as centres of the morphogenesis, which can give rise to organs such as roots, shoots and embryos [2]. The calli induced were hard and compact in nature. The green colour was observed among the calli, they were of the meristamoid units. The meristarnoids appeared as small shining nodular structures over the calli.

Callus depended on the genotype, the concentrations and composition of growth substances and number of in-vitro regeneration cycles undergone by the donor plant [4]. Different callus has developed to serve different purposes. Usually, in peach, white callus are involved in production of embryogenic cells or embryos [19] and green coloured callus are ultimately converted different organs [20]. Bhojwani and Razdan [15] also recorded firm and compact callus from stem internodes in peach. Petiole with nodal segment was found best explant for callusing in Laurel (*Laurus nobilis* L.) [21]. Similarly, apical shoot and shoot with internode were reported to be better over the leaf explant for callus induction purposes. Such types of variation in response to different explant type have also been reported earlier [22]. Callus was initiated from cut ends of green tender leaves and internodal segments of peach in MS medium.

Organogenesis and somatic embryogenesis are two different mechanisms, by which explants can regenerate the complete plants. The newly growing plant part (meristem) is the ultimate source of all tissues in the plant body, and these generative activities are supported by a pool of stem cells residing within the meristem. Thus, it is not surprising that strong activation of these meristematic activities leads to ectopic callus induction from explants. The stem cell is organizing centre of shoot meristems and is required to maintain stem cells in a relatively undifferentiated state [23].

According to Karp [24], the differences observed in callus proliferation occur because the explants can vary in their sensitivity to growth regulators and/or due to the differences in endogenous content of the hormones.

High concentrations of the auxins 2,4-D in the culture media are necessary for the induction of nodular embryonic structures in peach during the induction of somatic embryogenesis [11]. Number of authors previously reported SEM observation in different crop species callus and almost find similar type of observation [25,26] in perennial ryegrass and tall fescue callus; Popielarska et al. [27] endosperm derived callus of *Actinidia deliciosa* [28–30].

Number of literatures supports, the embryogenic callus has been characterized by the presence of whitish, compact and organized embryogenic sectors [31], or as described by Van-Le et al. [32], compact and white embryogenic bodies which were scored as somatic embryos. White, friable calli with nodular structures that gave rise to somatic embryos when transferred to appropriate medium [33]; and white, friable callus developing green meristematic centers, which later became somatic embryos [34]. These features occurred in cultures of peach produced by the series of treatments described in the above experimental results. The present results provide the first successful report of induction of embryogenic tissue in peach and establish the basis for further research on somatic embryogenesis of this species. Our results confirmed the effectiveness of certain auxin and cytokinin combinations in the induction and proliferation of callus. Characteristics associated with embryogenic callus, glossy, globular structures and elongated suspensor-like cells, developed after sub-culturing in fresh medium. Among various auxins tested, 2,4-D was more effective than IAA, IBA or NAA in producing somatic embryogenic calli. Similar type of result was obtained in our present study. The combination of 2,4-D and BAP appears to enhance embryogenic potential of callus.

Histological studies in pearl millet revealed the presence of small and richly cytoplasmic cells with starch containing cells and compact clumps were termed as embryogenic cells and they also observed the presence of elongated, thick-walled cells with large vacuoles in the young inflorescence cultures [35]. Similar results were also reported in the callus culture of *Etlingera elatior* [36] and SEM analysis of embryogenic callus of sugarcane [37] that shows the formation of pro-embryos from a group of embryogenic cells in the embryogenic callus. Detailed histological observations on initiations of morphogenesis in immature embryo and mature embryo of barley [38]. The similarity of structure characteristics observed in this SEM analysis supports the embryogenic potential of the globular callus induced in this study of in stem explant of *Labisia pumila* var. *Alata* [39].

5. Conclusion

The present SEM study clearly demonstrates that somatic cells from peach leaf and intermodal segment explants generated callus could develop into fully differentiated somatic embryos through the characteristic embryological patterns of differentiation.

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software, TSB and BC; validation, TSB, LR and AV; formal analysis, TSB, LR and BC; investigation, TSB; resources, PNS; data curation, TSB and BC; writing—original draft preparation, TSB and LR; writing—review and editing, LR and BC; visualization, AV; supervision, PNS; project administration, PNS; funding acquisition, PNS. All authors have read and agreed to the published version of the manuscript.

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Data availability statement: The data that support the findings of this study are available from the corresponding author, [TSB], upon reasonable request.

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Abbreviations

BAP	6-benzylaminopurine
GE	Globular Embryo
HS	Heart Shape
IS	Internodal segments
LD	Leaf disc
MS	Murashige and Skoog medium
SEM	Scanning Electron Microscope
SC	Spherical cell
2,4-D	2,4-dichlorophenoxyacetic acid

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