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Research Article

Somatic embryogenesis induction in four cassava landraces in East Java, Indonesia

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Abstract *Manihot esculenta* Crantz, commonly known as cassava, is a staple aliment that is a significant source of revenue for farmers. The embryogenic callus is crucial in the genetic engineering of various crop species, including cassava. Four cultivar cassava landraces from East Java were assessed for their ability to produce friable embryogenic callus (FEC) for protoplast isolation. In this study, four cassava cultivars; (Kaspro, Kuning, Gajah, and Gendruwo); were used to obtain FEC, which involved the culture of immature leaf lobes (ILLs) and apical buds (ABs) media containing MS supplemented with 33 μM picloram and 2 μM of $CuSO_4$ (M1) or MS supplemented with 50 μM 2,4-D and 2 μM $CuSO_4$ (M2). The highest FEC induction efficiency ranged from 72% to 57%, and the highest FEC number ranged from 4.7 to 3.7 with AB explants in media containing MS + 33 μM pilocram and 2 μM $CuSO_4$ (M1). On the other hand, the efficiency of somatic embryogenesis induction ranged from 67% to 53%, and the number ranged from 4.4 to 3.4. The efficiencies of FEC induction ranged from 48% to 42%, and the number ranged from 3.1 to 2.6 with AB explants in media containing MS + 50 μM 2,4-D and 2 μM $CuSO_4$ (M2); the efficiency of FEC induction ranged from 56% to 50%, and the value ranged from 3.6 to 2.4 with ILL explants. The FEC induction of the Gendruwo cultivar, which was examined using AB and ILL explants, demonstrated the lowest efficiency. Nevertheless, all four cultivars showed the ability to generate FEC, even though their effectiveness differed depending on the explant genotype and the applied media.

Keywords Cassava, FEC, Organogenesis, Somatic Embryo

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Introduction

Almost all tropical lowlands cultivate cassava (Manihot esculenta Crantz), a plant from the Euphorbiaceae family (Howeler et al. 2013). Since it is tolerant of unfavorable environmental circumstances, cassava has a high level of adaptation to poor soil, and has flexible harvest seasons, cassava plays a significant role in food security in many developing nations (Zhang et al. 2018). Cassava is a tropical plant that can thrive in various soil conditions and even adapt to barren soils (Priadi et al. 2008).

To enhance the genetic quality of cassava, genetic engineering as an alternative approach is used. When it comes to developing seeds that are resistant to pests and diseases or enhancing the quality of their tubers, genetic engineering for cassava has a great deal of opportunity to supplement conventional breeding procedures. According to Zhang et al. (2017), some genetic alterations of cassava include root production, post-harvest storage, and the creation of virus-resistant cassava as well as biofortification and starch modification for industrial uses and improved storage. The continual production of transgenic plants relies on the frequent and effective production of excellent FEC for use in agrobacterium-mediated transformation experiments, which is the key to cassava transformation. Using constitutive promoters like CaMV35S and NOS is a frequent approach to enhance particular cassava features. In order to effectively develop the crop utilizing biotechnological equipment, it is crucial to comprehend and prioritize the distinct biological characteristics and economic attributes of cassava.

Conventional cassava breeding often undergoes problems, including high heterozygosity and low fertility. In addition, conventional breeding results in short growth cycles so seed production is very limited. Vitro cassava research is focused on clonal propagation and genetic transformation

technology to obtain the desired traits. Protoplast fusion is an effective complement method to overcome the constraints of generative plant propagation as well as an alternative to solving problems in conventional breeding. Protoplast regeneration is a prerequisite for protoplast fusion. FEC originates from a single cell and multiplies rapidly so that the tissue has a reduced risk of chimerism.

Regular drawbacks with conventional cassava breeding include excessive heterozygosity and low fecundity. Moreover, conventional breeding produces short growth cycles, which results in a low seed yield. For the aim of gaining the necessary features, vitro cassava research focuses on clonal propagation and genetic transformation technology. To overcome the limitations of generative plant reproduction and as an alternative to resolving problems in traditional breeding, protoplast fusion seems to be an efficient supplement method. However, protoplast regeneration must initially occur for protoplast fusion to happen. FEC develops from a single cell and multiplies quickly, decreasing the probability to chimerism in the tissue. The prolonged in vitro tissue culture process could lead to somaclonal diversity. For all processing methods, crumbly embryogenic callus has been utilized as the precursor material. Somatic cotyledons and FEC are induced from somatic embryos and these explants are exploited for transformation. Stamp and Henshaw (1982) were the ones who originally defined the somatic embryogenesis of cassava.

Protoplast electrofusion was carried out by Wen et al. (2020) using mesophyll protoplasts from cultivar SC8 and callus protoplasts from cultivar TMS60444. The fusion products were grown approximately one to two months in a protoplast growth media (TM2G) with progressive dilution. Compact calli that were produced from protoplasts were put into a suspension culture medium (SH) for suspension culture. The cultured products successively grew into embryos, mature embryos, shoots on somatic embryo emerging medium (MSN), embryo maturation medium (CMM), and shot extension medium (CEM). The operation was effective in producing some new cassava germplasm, along with having resulted in the development and validation of a protoplast electrofusion methodology.

The objective of this research was to figure out the ideal explant size and media composition for inducing embryogenic calluses, primarily friable embryogenic calluses (FEC), used for rapid propagation and as a target for protoplast fusion-mediated genetic transformation.

Materials and Methods

Plant materials

This study included four varieties of cassava, Kaspro, Kuning, Gajah, and Gendruwo with freshly developed stem-apical buds that measured 2 cm. The shoots were extracted from the parent plant, transported to the lab, and sterilized there by submerging them in a solution containing 1.5% sodium hypochlorite and 0.01% Triton X-100 for five minutes. Three primordial leaves from the immature leaf lobes (ILL)-shoots were removed aseptically and moved into a test tube (15 × 2.5 cm) filled with 10 ml of solid MS medium and 0.1 mgL^{-1} of benzyl adenine (BA). For 16 hours, the explants were incubated inside the growth cabinet at 26°C with 25.3 μmol $m^{-2}s^{-1}$ of intense light coming from white fluorescent lamps, followed by 8 hours of complete darkness.

Embryogenic tissue induction

Two-centimeter shoots extracted from the mother plant were sterilized for five minutes by putting them in a solution of 1.5% sodium hypochlorite and 0.01% Triton X-100. They were then transplanted onto 10 ml of solid MS media and given 0.1 mgL^{-1} of benzyl adenine (BA). The explants were transplanted into a petri dish containing 25 ml of solid MS medium supplemented with 2% sucrose, 0.5 mgL^{-1} copper sulfate, and picloram concentration of 15 mgL^{-1}, solidified with 0.6% agar under aseptic circumstances in order to induce somatic embryogenesis (SE).

The experiment was executed with a completely randomized design (CRD). A total of 90 explants were accumulated as each treatment was carried out three times (30 explants in each replication), and the data were gathered three weeks after the beginning of culture. Using the SAS application (System for Windows, version 8.00), the data were examined using analysis of variance (ANOVA). The Tuckey test was then employed to compare the means using a minimum significant difference (SMD) at a 5% level.

Using small forceps and a scalpel, immature leaf lobes and apical buds were removed from each mother plant at the age of five weeks and placed in 8 Petri dishes per cultivar supplemented with 33 uM picloram, 50 uM 2.4-D, 3% sucrose, and 0.3% gelrite as medium M1 and medium M2, respectively. Ten young leaf lobe explants and ten apical bud explants were used in each petri dish for each treatment. After two weeks in culture, the explants were transferred to fresh medium. In six weeks, the formation

of embryogenic tissues from leaf lobe explants was assessed. Each treatment's frequency of explants producing Friable Embryogenic Callus (FEC) was recorded, and its production was measured using a scale from 0 to 10.

Histology

Histological observations were carried out to investigate embryonic development starting from the initiation of callus, embryonic callus. The histological procedure included tissue fixation with FAA. Then, the samples were dehydrated with alcohol with gradual concentrations starting at 70%, 80%, 90%, then 100%. Samples were cleared using a xylol: alcohol mixture starting at a ratio of 1:3, 1:1, and 3:1. Next, infiltration was carried out using paraffin, and using a rotary microtome cutting (Rotary Microtome YD-202A MRC) with a thickness of 8-12 μm. The next stage of gluing the cutting tape was prepared and stained using 1% of Safranin (O-C.I. 50240- Merck). Mounting using Canada balsam was the final stage of microscopy observation by Leica EZ4 HD and Optic Lab.

SEM

Three-week-old friable cells cell culture supply were identified through a SEM analysis. The callus was taken out from the petri dish and put into a fixative solution containing 2% of glutaraldehyde & 2% paraformaldehyde in 0.1 M of cacodylate buffer with a pH of 7.2, and 3% of sucrose. After that, the fixed tissue was washed using 0.1 M of cacodylate buffer. Ethanol was used for dehydration in stages. The dried samples were observed using an FEI QUANTA 450 Scanning Electron Microscope.

Results and Discussion

The effect of media types and cultivar on the FEC frequency

The highest average number of FECs (approximately 4.8) were found to be produced by apical shoot explants of the Kaspro and Kuning cultivars employing M1 MS+ $CuSO_4$ 2 uM and Picloram 33 uM, as well as ILL explants of the Kaspro cultivar (around 4.4) and Gajah cultivar (around 3.9). In comparison to M1 medium, M2 MS+ $CuSO_4$ 2 uM, 2,4D 50 uM produced substantially lower FEC counts in the four cultivars. The cultivar Kaspro had the greatest FEC with ILL explant (4.8), and cultivar Gendruwo produced the least value (1.3). We have recorded the values of FEC with BA explant in Gajah, Kuning, and Gendruwo cultivars in the M2, which ranged from 3.7, 4.6, to 1.2. For the M1 and M2 media, the average FEC Gendruwo cultivar bore the lowest yield.

Frequency analysis revealed that the Kaspro cultivar (72%) with the most FEC (Table 1) with AB donor explants, and Gendruwo cultivar (21%) with the lowest, were grown in medium M1. In addition, the Gajah and Kuning cultivars scored 59% and 53%, respectively. When using donor explants ILL on the same M1 media, the frequency of FEC induction varied as 67% with the Kaspro cultivar and 19% with the Gendruwo cultivar. The Gajah and Kuning cultivars respectively scored 59% and 53%. The FEC with donor explants ILL in the medium M2 ranged from 50% in the Kaspro cultivar to 15% in the Gendruwo cultivar, whereas the Gajah and Kuning cultivars had the frequencies of 56% and 37%. The frequencies of FEC involving donor explants' apical buds, on the other hand, ranged from 48% in the Kaspro cultivar to 12% in the Gendruwo cultivar. Furthermore, we have assessed respectively 40% and 42% in the Gajah and Kuning ones.

The quantity of FEC created per explant planted was used to evaluate the efficacy of FEC synthesis. Efficiency and the frequency of FEC formation were related. It displayed the same pattern when the FEC frequency and the FEC formation value were linked (Fig. 2). High efficiency values correspond to high FEC frequency percentages. The percentage of FEC in the M1 medium Kaspro variety was 72%, and the FEC formation value was 4.7. With a frequency of 70% (close to the Kaspro cultivar), and an efficiency value of 4.6, the Kuning cultivar possessed a significant proportion of FEC. The Gendruwo cultivar showed a low value percentage. In the M2 medium, percentages and efficiency figures followed a similar pattern.

Table 1 Effect of the media and explants on the frequency of FEC induction

Frequency of FEC induction (%)	Kaspro		Gajah		Kuning		Gendruwo	
	Media		Media		Media		Media	
	M1	M2	M1	M2	M1	M2	M1	M2
AB	72	48	57	40	70	42	21	12
ILL	67	50	59	56	53	37	19	15

Historically, research on cassava has lagged behind those of crops grown in temperate climates (Olsen and Schaal 1999). Traditional breeding has contributed significantly to the genetic advancement of this crop, allowing for the introduction of beneficial traits such as bacterial and viral resistance into the cassava germplasm (Ceballos et al. 2007; Chávez et al. 2005; Morante et al. 2010; Rudi et al. 2010). Conventional breeding methods have a number of drawbacks, most notably the crop's heterozygous character, which makes it challenging to determine the true breeding potential of parental lines. Besides that, a gronomically significant hereditary features are also largely unexplored (Ceballos et al. 2004; Nassar and Ortiz 2010; Olsen and Schaal 1999).

Cassava improvement can be challenging due to the difficulties and the fact that not all cultivated genotypes are adaptable to breeding and generating blooms. Consequently, genetic engineering has emerged as the preferred technique for improving cassava. This technique essentially involves transforming friable embryogenic calluses using agrobacterium (González et al. 1998; Zhang et al. 2001). Each cassava cultivar employed in this study responded to the medium in a remarkably different way. Axial bud and ILL explants were employed for planting, and both had varied outcomes. Making and preparing cell cultures and pallets that will be used for protoplast isolation in subsequent experiments was the sole objective of this research.

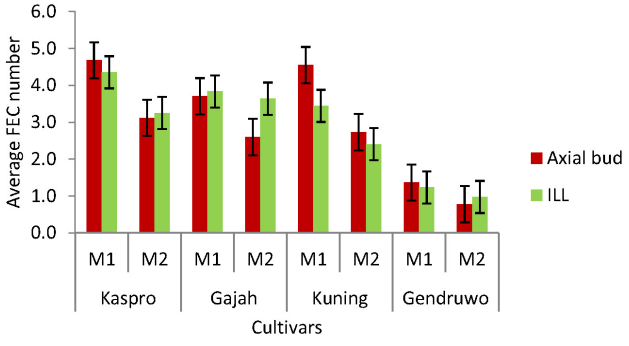

Fig 1 Effect of the media and the substances picloram and 2,4-D on the FEC number

The results revealed that the tested cassava cultivars might produce structured friable embryogenic callus (FEC). Overall, the findings showed that the Kaspro (Fig. 2 B, C) and Kuning (Fig. 2 A) cultivars produced the most FEC, followed by the Gajah Fig. 2 D) and Gendruwo ones (Fig. 2 E). Further, the findings showed that different cultivars responded differently to in vitro settings. In the past, 2.4-D and Picloram were added to the media, resulting in reports on the the FEC synthesis. Auxins have widely been utilized to induce cassava somatic embryos (Li et al. 1996; Taylor et al. 2001). In our example, a 50 µM concentration of Pic and 2.4-D was more successful at generating somatic embryos than a 33 µM concentration. Picloram has consistently been proven to be more successful in encouraging embryo development in cassava cultivars from Africa (Ng and Adeniyi 1994; Raemakers et al. 1993; Rossin and Rey 2011), South America (Feitosa et al. 2007), and Asia (Li et al. 1998; Saelim et al. 2006).

Explant types (such as ILL or AB), auxin types (such as picloram, and 2.4-D), and auxin concentration are all factors that affect a cassava genotype's capacity to produce somatic embryos or Friable Embryogenic Callus. We demonstrated that cultivar and auxin had an impact on the

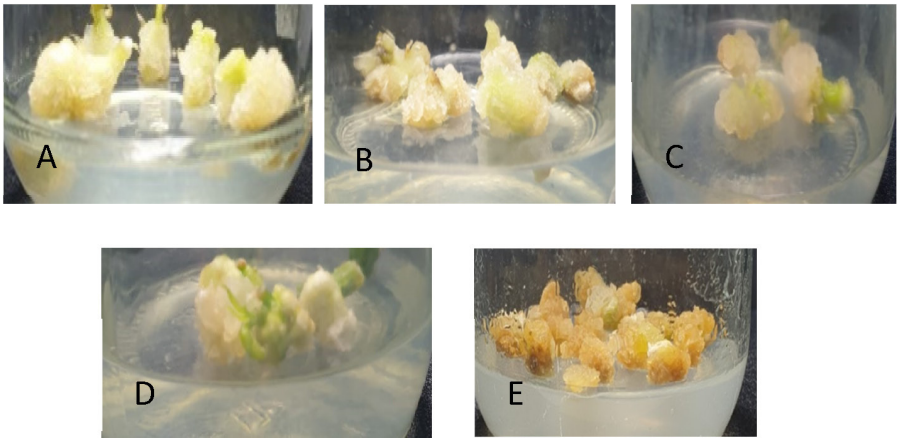

Fig 2 FEC induction from immature leaf lobe (ILL) and apical bud (AB) explants. A. AB explant of the Kuning variety on MS + copper sulfate and picloram (3 mgL^{-1}). B. AB explant of the Kaspro variety on MS + copper sulfate and picloram (3 mgL^{-1}). C. AB explant of the Kaspro variety on MS + copper sulfate and picloram (9 mgL^{-1}). D. AB explant of the Gajah variety on MS + copper sulfate and picloram (3 mgL^{-1}). E. AB explant of the Gendruwo variety on MS + copper sulfate and picloram (3 mgL^{-1})

frequency of somatic embryogenesis and the volume of embryos produced per explant, indicating a genotype-auxin interaction effect as described by several other researchers (Feitosa et al. 2007; Rossin and Rey 2011; Saelim et al. 2006). C ytokinins are crucial in the control of meristem morphogenesis and function, and along with auxin, they influence the development of plant shoots and roots (Werner et al, 2001). Consequently, cytokinin activity affects the activity of apical shoot meristems. Recent studies have shown that meristem activity and size are decreased by cytokinin depletion (Werner and Schmülling 2009). Studying the potential of additional auxins like picloram and 2.4D is necessary for enhancing the somatic embryogenesis system in cassava, particularly in cases when the plant's embryogenic potential is unclear. To promote somatic embryogenic competence, auxin is a crucial plant growth regulator, according to Gaj (2004), Lincy et al. (2009), and Danso et al. (2010).

An apical-basal axis is formed as a part of the embryonic process to transmit auxin via the PIN protein. While basal cells create suspensors, apical cells lead to the pro-embryo. PIN7 is present in the basal cells of phase 2 embryonic cells to convey auxin to apical cells. Auxin builds up throughout the pro-embryo as a result of PIN7 being present on the suspensor cells' apical membrane following two rounds of cell division. A WUS induction in the embryo may be connected to auxin accumulation in the apical meristem. When PIN7 is polarized in the 32-cell phase, auxin is delivered to the suspensor cells to create the hypophysis as the embryonic root (Friml et al. 2003). Nonetheless, this research has looked into the possibility of a connection between the frequency and the quantity of embryos produced. The amount of somatic embryos and their frequency showed a strong positive association. This indicates that proembryogenic mass conversion to different somatic embryonic developmental stages is highly efficient for the majority of cassava cultivars.

SEM

A firm, creamy callus was formed from an apical bud-ILL after two weeks in the induction media (Fig. 3a). Then, it was detached from the stems-apical buds for SEM examination and histological investigation. Each of the four cultivars' calluses had a similar appearance, with round forms and yellowish calluses on all four. During the SEM investigation, the callus from each cultivar was examined. For the Kaspro (Fig. 3b) and Kuning cultivars, a distinctive embryogenic spherical shape and a densely packed structure containing dense cells were observed. In contrast, the Gendruwo callus consisted of long tubular irregular cells, showing the transition from a membranous to a fibrillar structure (Fig. 3c, d). As seen in callus Gajah, the SEM investigation of the callus surface revealed the existence of a discontinuous amorphous layer outside the outer cell wall (Fig. 3e, f). This substance formed a network-like structure that united the embryogenic and non-embryogenic callus cells by covering their surfaces. Furthermore, trichomes appeared on all embryogenic and non-embryogenic callus surfaces (Fig. 3g, h).

The maturation and regeneration of new plants are important, and they can be initiated by callus inductions to obtain somatic embryogenesis. The initial process of callogenesis of cassava cultivars Kaspro, Kuning, Gajah, and Gendruwo was used for primary and somatic embryogenesis callus analysis using histological analysis and

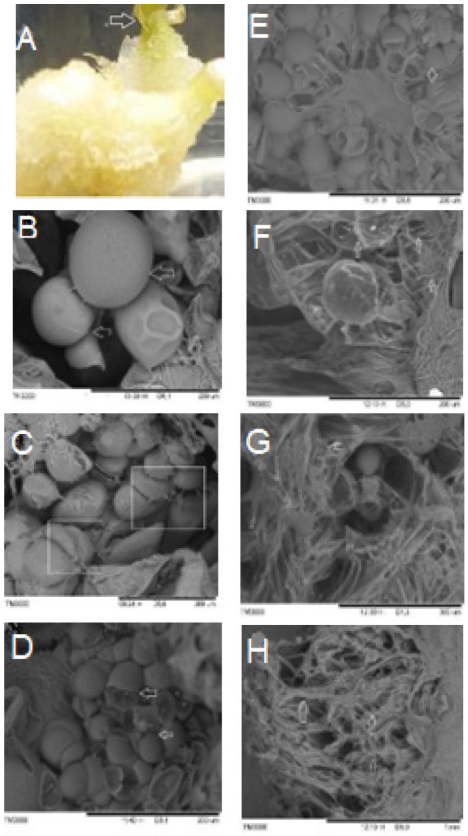

Fig 3 Development of the cassava callus and SEM results. A. Callus (cal) developed from the stem apical buds with the remaining explants. B. Spherical structure (black arrow) of compact calluses. The parenchyma cells of the callus consist of a few small cellular clusters. C. White boxes highlighting the fetal structure. D. Closed calluses with membrane structures (white arrows). E. Diamond showing the transition from the membrane to fibrillar structures. F. White arrows indicating the formation of a thick band by fibrils. Many trichomes are visible on the callus; note the length of cells (black arrow) that produce trichomes (G, H)

SEM. Fig. 1 demonstrates how the growth and development of cells of various genotypes differs depending on the genotypes of the culture media. Thus, it is necessary to adjust the media to the genotypes to be cultured (Hiej and Komari 2008). Because the development of embryogenic calluses depends on the interaction between the genotypes and culture settings, media with varying nutritional contents and growth regulating chemicals might promote the growth of FEC genotypes Kaspro and Kuning cultivars. The observation results of callus culture are in line with the observation on the callus culture of Swarma and Mashuri rice cultivars (Pravin et al. 2011). The results showed that callus induction is higher by 49% and 71% on modified MS media (Murashige and Skoog 1962) compared to basal MS media.

In all four cultivars, an extracellular matrix consisting of fibrils was found in the SEM observations (Šamaj et al. 1995). Somatic embryos begin with a callus formation on the explant's surface with the callus being clear yellow, growing fast, and forming dense granules in the middle of the surface (Zhao et al. 2008). These granules come from epidermal and sub-epidermal cells (Meilasari and Iriawati 2016). You can also see a similar structure in corn plants. Similar findings have been obtained by other researchers in Triticum aestivum (Konieczny et al. 2005; Pilarska et al. 2007) and Brassica napus (Namasivayam et al. 2006). Although the non-cellular nature of the ECMSN [Extracellular matrix surface network] was studied using SEM, observations conducted by Verdeil et al. (2001) and Konieczny et al. (2005) revealed that the nodular cassava callus segments had a smooth surface coated by a membranous layer.

Histology

We examined two-week-old calluses during histological analysis. Parenchyma cells made up the main calluses that emerged from the apical buds (Fig. 4a-c). The outermost part of the calluses consisted of closely packed meristematic cells, of which the outer (callus margin) was isodiametric, non-meristematic, densely stained areas were arranged into small units (Fig. 4a, c). Mature calluses where the cells were larger and the position between cells was not dense, were in the meristematic part. Tiny units were particularly numerous in the peripheral areas of the callus (Fig. 4b). In addition, this area of the callus was made up of a combination containing various callus tissues. The histological section of the meristematic area in mature calluses showed that the cells were actively dividing, making them appear dense and dark. These cells appeared to be located among the non-meristematic primary callus cells (Fig. 4c).

The histological study we conducted shows that the spaces between cells of the fibril or reticular callus tissue are less dense as indicated in Fig. 3c, this is in accordance with the results of a study by Ovečka and Bobák (1999) which finds that during somatic embryogenesis, dense spider webs will form between the big intercellular spaces. Furthermore, according to Sawidis et al. (1991), plant cells are polysaccharide polymer complexes which in certain cases are found in microfibrillar tissue textures.

The histology results of the 2-week-old callus show the meristematic area where small cells are densely packed. In the outer part, in which a callus edge is a non-meristematic area, appeared initiation of calluses and a little embryogenic area (callus) that formed a globular at the end. Meanwhile, in the histological section of an adult callus, the cells were more giant and the position between the cells was not dense. We found that the histological section in a mature callus was a meristematic area where the cells actively divided to appear dense and dark. These cells appeared to be located between the non-meristematic primary callus cells.

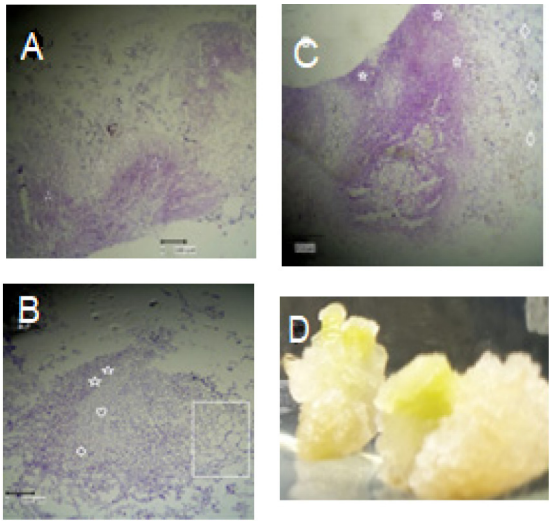

Fig. 4 Histology of 2-week-old calluses from various cassava cultivars. A. Histological section of a callus induced at two weeks of age; the dark part (☆) is a meristematic area where small cells are densely packed, and the outer part (callus edge) is a non-meristematic area (◇). The induction of calluses and a small embryogenic area (callus) that forms a globular structure at the end could be observed. B. Histological section of an adult callus where the cells are larger and not dense. The meristematic part (☆) is curved on the left side, there is a tissue-like callus in the middle (□), and there are small portions of non-meristematic callus (◇) on the right side. C. Histological section of a meristematic area of a mature callus where the cells are actively dividing such that they appear dense and dark (☆). These cells appear to be located between the non-meristematic primary callus cells (◇). D. Calluses 2-week-old cassava

According to Tomaz et al. (2001), high multiplication power in media containing sucrose causes an intensification in the number of calluses. Before the epidermal callus segment, for example, tissue develops, then the surface will be protected by a layer of mucus. In the cell-to-cell adhesion process, pectin compounds play an important role. According to Popielarska et al. (2006), due to the role pectin plays in cell-to-cell adhesion, it is conceivable that the surface layer is involved in the integration and identification of morphogenic cells in multicellular nodular structures. The induced cell surface of the explants will be covered by a fibrous material that appears due to the induction of the morphogen site; this phenomenon occurs in several plants cultivated in vitro.

Before the tissue resembling the epidermis grows, the surface layer of mucus may shield the surface callus segments. Pectin's function in cell-to-cell adhesion raises the possibility that the surface layer contributes to the integration and identification of morphogenic cells in multicellular nodular structures. The emergence of the fibrous material covering the cell surface of the induced explants is tied to the induction of the morphogen in some in vitro-cultivated plants.

Extracellular matrix surface tissue (ECMSN), also known as surface tissue, has been observed during somatic embryogenesis in a number of plants, including Cocos nucifera (Verdeil et al. 2001), Drosera spathulata (Bobák et al. 2003; 2004), and Coffea arabica (Sondahl et al. 1979). This is because certain in vitro conditions can cause stress on explants caused by the formation of ECMSN.

These findings may be practical for various cassava cultivars and may lead to novel methods of exploiting cassava calluses by cutting 2-week-old calluses. Furthermore, the results of this study can be developed to help produce more embryogenic calluses using less calluses in a shorter time.

Conflict of Interests

The author of this research identifies no potential conflicts of interest.

Acknowledgement

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