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Research Article

Somatic embryogenesis in two cassava (*Manihot esculenta* Crantz) genotypes

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Abstract

Plant breeding through hybridization in cassava is facing a problem due to inconsistent flowering, and also the donor genes controlling superior traits are limited. An alternative method of breeding is through genetic transformation, and regeneration via somatic embryogenesis is promising route to achieve this. As somatic embryogenesis in cassava is genotype-specific, in the present study a protocol has been developed for UJ-3 and BW-1 genotypes. Immature sterile leaves from 7-10 days axillary shoots in a pre-condition medium were used as an explant. Leaves were inoculated on Murashige and Skoog (MS) medium containing picloram (0.0, 7.5, 10.0, 12.5, and 15.0 mg/L) and 1-naphthalene acetic acid (NAA, 6 mg/L) for induction of somatic embryos (SEs). Genotype BW-1 showed best results as early callus formation time i.e. 8.04±0.32 days after induction (dai) compared to UJ-3 (8.67±2.13 dai). The callus fresh weight (0.64 g) was also higher in BW-1 than UJ-3 (0.38 g) after 4 weeks in callus induction medium (CIM), and the callus formation ranges between 85.19±3.70 to 96.30±3.70% for both genotypes. Subculturing embryogenic callus to MS+CuSO4 (4 µM) + picloram (6 mg/L) +NAA (0.5 mg/L) (SK1 medium) germinated maximum SEs in BW-1 (46.56±36.86), whereas the number was less for UJ-3 (11.89±11.90). Further, shoots were developed from green cotyledons followed by hardening and acclimatization of plantlets.

Keywords: BW-1; cassava; NAA; picloram; UJ-3

Introduction

Cassava (*Manihot esculenta* Crantz) is a tropical and subtropical edible plant which potentially support food security programs, even the Indonesian minister of Agriculture stated that cassava as one of the potential local food commodities to be developed in the mid of the Covid-19 pandemic apart from sweet potato (*Ipomoea batatas*), taro (*Colocasia esculenta*), stink lily (*Amorphophallus muelleri*) and sago (*Metroxylon sago*). Carbohydrate content in fresh weight of cassava root ranges from 32-35% and 80-90% in dry matter. However, starch content is most important as it can be used as raw material for production of ethanol and other industries

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(Uchechukwu-Agua *et al*., 2015). It has been well documented that the starch derived from cassava is more better for conversion to biofuels as compare to other starch such as corn, wheat, and rice (Krajang *et al.*, 2021). Furthermore, the plant has been reported as a major source of calorie in country like Africa (FAO, 2020) and major source of starch in some Asian (Howeler, 2012) as well as in American countries (Aristizabal *et al.*, 2017). However, in nature it encountered different biotic and abiotic stresses such as drought, pest, weed, and disease caused by bacteria, fungi and virus. In addition, there are also problems with the quality of root production, the low protein content (<2%) and high cyanide content about 120 to 1941 HCN equivalent per kg root dry weight (Iglesias *et al.*, 2002). Further, rapid physiological deterioration of the root after harvest lead to limited shelf-life (Uchechukwu-Agua *et al*., 2015). Thus, there is urgent need to improve the crop genetics which can be done via conventional breeding through sexual hybridization to develop cassava superior variety (Ceballos *et al.*, 2012). However, this technique has drawback as it takes long time in breeding cycle (about 8 years) and low rate of multiplication (Ceballos *et al.*, 2020), highly heterozygous, allopolyploidy, and irregular flowering time (Danso and Ford-Lloyd, 2002).

In vitro regeneration through somatic embryogenesis could be useful for genetic manipulation (Elegba *et al.*, 2021) and genome editing (Odipio *et al.*, 2017). Somatic embryogenesis in cassava has been reported previously (Danso and Ford-Lloyd, 2002; Rossin and Rey, 2011; Ma *et al.*, 2015; Mongomake *et al.*, 2015; Raemakers *et al.*, 2006; Danso and Elegba, 2017; Susanti *et al.*, 2017; Pais, 2019). However, it is observed that somatic embryogenesis in cassava is affected by many factors such as source of explant, medium composition, culture environment, and molecules secreted by plant into medium (Nic-can *et al.*, 2015). It is also difficult to regenerate plant from callus as it is genotype-dependent (Opabode *et al.*, 2013), therefore effect of plant growth regulators on *in vitro* morphogenic response for each genotype must be investigated. Cassava genotypes UJ-3 and BW-1 are most preferred genotypes and widely planted by Indonesian farmers, especially in the province of Lampung (Sholihin, 2021), nonetheless the reports are scanty on somatic embryogenesis for these genotypes. Another factor which affects somatic embryogenesis in cassava is plant growth regulators (PGRs), and picloram has been reported as the best growth regulator used for inducing somatic embryo in cassava besides 2,4-dichlorophenoxyacetic acid (2,4-D) (Mongomake *et al.*, 2015; Danso and Elegba, 2017; Susanti *et al.*, 2017).

Thus, present study aimed to investigate efficient plant regeneration through somatic embryogenesis in farmer preferred two Indonesian cassava genotypes using immature leaf lobes explant for the first time.

Materials and Methods

Plant materials and sterilization

UJ-3 and BW-1 cassava genotypes were used as sources of material for establishment of *in vitro* cultures. The stem cuttings (25 cm) of these genotypes were obtained from farmers in Tanjung Bintang, South of Lampung, Indonesia, and they were grown in the polybags and maintained in the greenhouse. Three nodes from the top of two-weeks-old axillary shoots were excised, then all leaves were removed and sterilized by cleaning them under running tap water for 30 min. After that, they were washed with detergent solution, rinsed with distilled water and the cleaned shoots were cut into 5 cm length. The next sterilization procedures were performed in laminar air flow cabinet (Bio Chamb, Indonesia) by soaking the shoots in NaOCl (20% v/v) with 2 drops of tween-20 per 100 mL solution for 15 min and then washed 3 times with sterile distilled water. Subsequently, the shoots were soaked in ethanol (70% v/v) solution for 1 min followed by washing with sterile distilled water 3 times. Eventually, the shoots segments were cut to 1-2 cm where each shoot contained one node.

Callus induction

The sterile shoots were grown on Murashige and Skoog's (MS, 1962) basal medium (Phytotech, Lenexa, USA) without PGRs and incubated in a growth room. The immature leaf lobes (5-10 mm) from 7-10 days old plants were used as the explant and cultured on callus induction medium (CIM) for callus inducing which consist of MS basal medium augmented with sucrose (4% w/v) (Sugar Group, Lampung, Indonesia), CuSO4 (4 μM), picloram [0 (M0), 7.5 (M1), 10.0 (M2), 12.5 (M3), and 15.0 (M4) mg/L] and 1-naphthaleneacetic acid (NAA, 6 mg/L). pH of the media was set 5.8 using pH meter (Hanna instrument Indotama, Jakarta, Indonesia) and Bacteriological agar (0.8% w/v) (Oxoid, Hampdshire, UK) was used as solidifying agent. The mixed media was boiled and transferred into a 250 mL culture vessel with a 25-30 mL medium solution for each vessel followed by autoclaving (Tomy, Japan) at 121 °C with pressure of 1.2 kg/cm^2 for 15 min. Then, they were cultured at continuous dark conditions with 23-25 °C for 4 weeks. The effect of picloram concentration on callus formation was calculated by scoring the callus proliferation rate between 0-4.

Somatic embryo germination and maturation

To develop the embryos, 0.2 g embryogenic callus was taken from the callus developed in the first step (M0-M4 of CIM), separated from explant and transferred into somatic embryogenesis development medium (SEDM) consisted of M0-M4 and SK1 [MS medium, sucrose (4%), CuSO4 (4 μM), picloram (6 mg/L) and NAA (0.5 mg/L)]. Then, cultures were transferred to the same growth room condition. The somatic embryos (SEs) were examined using Olympus binocular microscope (Olympus, Tokyo, Japan) and scanning electron microscope (SEM) after 4 weeks.

Development of somatic embryos, plantlet hardening and acclimatization

For development of SEs, matured SEs from SEDM were selected for further developed into plantlets. MS basal medium without BA and with BA (0.2 or 0.4 mg/L) were used as a development media and SEs were transferred for 4 weeks. Further, the green shoots were transferred into MS medium with or without 1% activated charcoal for another 2-3 weeks to convert them into normal plantlets. Acclimatization process was performed by transferring of plantlets with defined shoots and roots to small pots containing sterile mixture of soil:cocopeat (1:1 w/w) for 4 weeks in culture room. Subsequently, the survived plants were transferred to polybag (30×30 cm) and maintained in the greenhouse for acclimatization.

Culture conditions

All the cultures were kept in growth room with a temperature of 23-25 °C under 24 h of light with photosynthetic photon flux density (800-1000 lux) provided by white fluorescence tubes (Philips, Netherlands). The culture for primary callus induction and somatic embryo development were incubated in dark condition with a temperature of 23-25 °C.

Experimental design and statistical analysis

The experiments were done in a completely randomized design with factorial arrangements namely genotype as the first factor consisting of UJ-3 and BW-1, and concentration of picloram as the second factor (0.0, 7.5, 10.0, 12.5, and 15.0 mg/L). Each treatment was repeated five times, each replicate consisted of three bottles with three explants for each bottle. The data was analysed using ANOVA and the significant means were further analysed by Least Significant Difference (LSD) at the 5% level.

Results

Callus induction

Immature leaf lobes were inoculated on callus induction medium (CIM) to investigate the effect of picloram on time of callus formation, callus morphology and percentage of induction in two cassava genotypes. The results suggested that picloram concentration or genotype showed no significant effect on callus induction time or frequency of callus formation. Primary callus formation started within 8-13 days after inoculation on all treatment media with the percentage of callus between 85.19 to 96.30% (Table 1). The callus formation initiated either at the edges of explant (Figure 1A) or from the midrib after the first week along with significant increase in explant size (Figure 1B). The explants of both genotypes continued to differentiate callus and formed compact as well as off-white colour embryogenic type of callus after 3 weeks (Figure 1C). Whereas root formation occurred from both genotypes in the control medium (Figure 1D). It was noted that addition of picloram affected the development of callus per explant 3 weeks after inoculation (Figure 2).

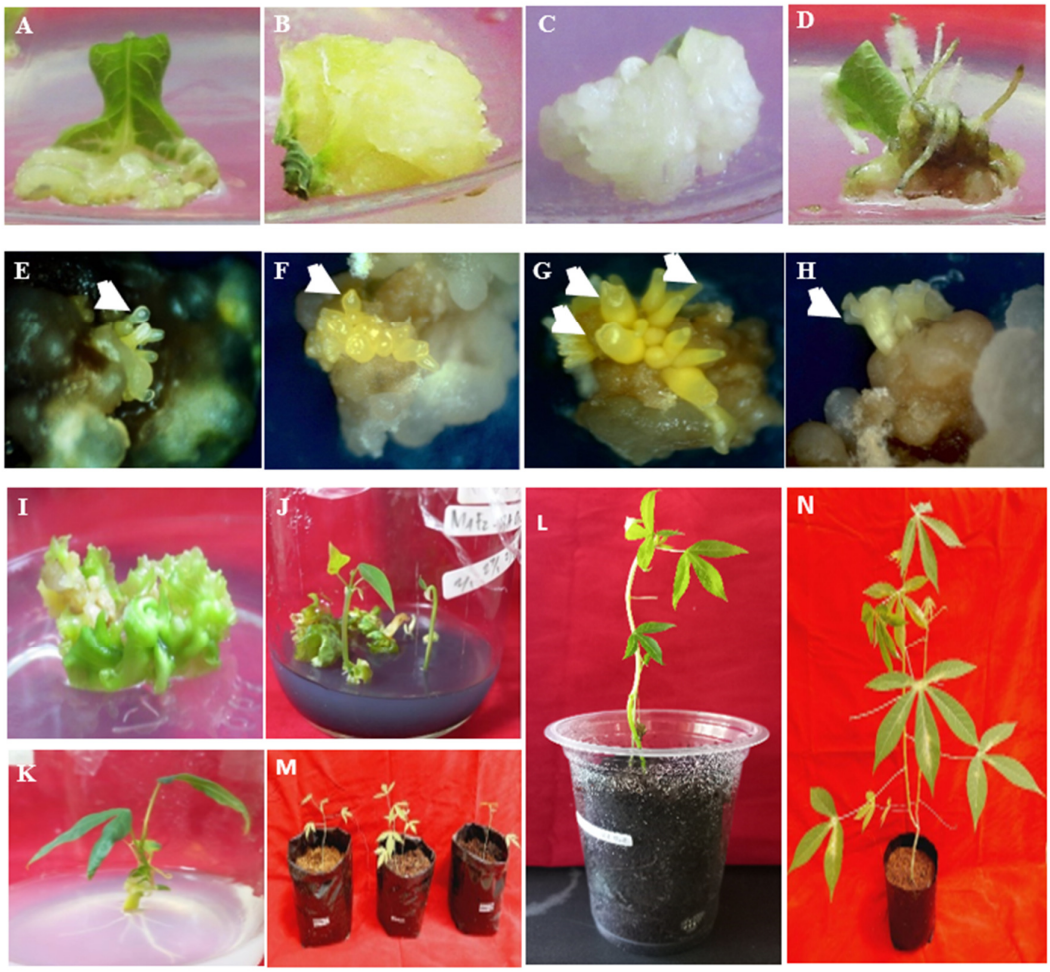

Figure 1. Regeneration of two cassava genotype UJ-3 and BW-1
Primary callus after 1 week from: (A) edge of explant and (B) midrib, (C) embryogenic callus after 3 weeks, (D) callus with root formation, (E-H) somatic embryo stages after transfer of embryogenic callus on SEDM after 4 weeks: (E,F) globular stage, (G) elongated globular, heart and torpedo stage and (H) cotyledon stage, (I) cluster of green cotyledon SEs and shoot buds on plant regeneration medium, (J) shoot derived from green cotyledons in presence of activated charcoal, (K) plantlet derived from SE after 3 weeks, (L) acclimatized plantlet in soil:cocopeat (1:1) after 4 weeks and (M,N) acclimatized plants in greenhouse after: (M) 2 months and (N) 4 months

Table 1. Effect of different concentrations of picloram on callus formation from two genotypes of cassava (4 weeks)

Medium	Genotype	Callus Induction Time (days)*	Callus Induction (%)*
M0	UJ-3	9.89±0.33	85.19±3.70
	BW-1	13.44±1.03	92.60±3.70
M1	UJ-3	10.00±0.99	96.30±3.70
	BW-1	11.41±0.26	88.89±6.42
M2	UJ-3	9.74±0.65	96.30±3.70
	BW-1	8.04±0.32	88.89±6.42
M3	UJ-3	8.67±2.13	88.89±6.42
	BW-1	9.15±2.19	96.30±3.70
M4	UJ-3	9.22±1.17	92.60±7.41
	BW-1	10.78±0.62	92.60±3.70

M0 = 0 mg/L picloram, M1 = 7.5 mg/L picloram, M2 = 10 mg/L picloram, M3 = 12.5 mg/L picloram, M4 = 15 mg/L picloram

*Data represents Mean±SE (n=30). Means are not significantly different ($p \leq 0.05$) according to Least Significant Difference (LSD)

The highest callus formation was recorded in both genotypes at two concentrations i.e. 7.5 and 10.0 mg/L of picloram, and it was 96.30±3.70% for UJ-3 and 88.89±6.42% for BW-1 with 10.00±0.99 and 8.04±0.32 callus induction days for UJ-3 and BW-1, respectively. The callus covered 26 to 50% of explants (Figure 2) and the weight of callus suggested that the genotype BW-1 showed significant callus proliferation as compared to UJ-3 (Figure 3).

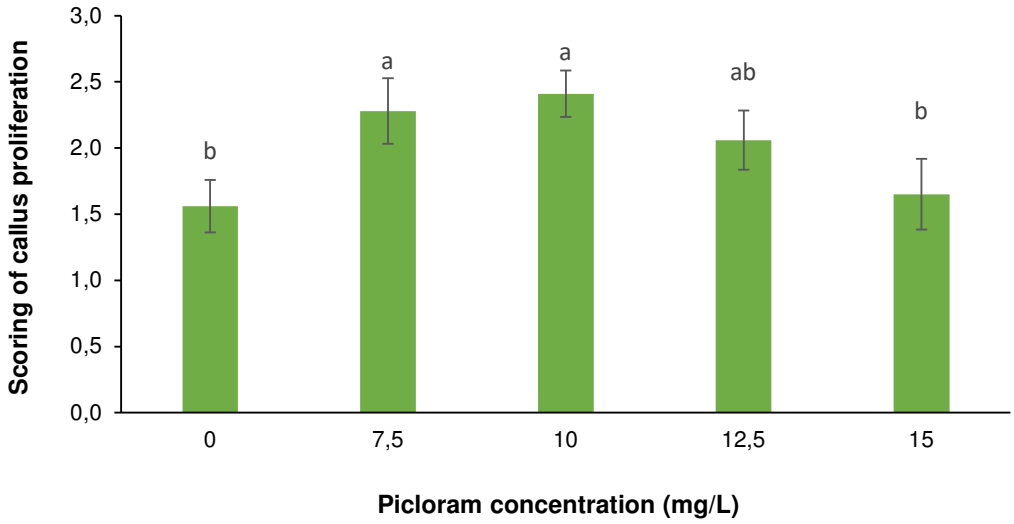

Figure 2. Effect of picloram concentrations on callus development after 3 weeks of induction. Scoring of callus proliferation- 0: no callus, 1: callus formation up to 25%, 2: callus formation between 26-50%, 3: callus formation between 51-75% and 4: callus formation more than 75%

Each bar represents mean values (n=30) and error bar as standard error. Bars having same letters are not significantly different ($p \leq 0.05$) according to Least Significant Difference (LSD)

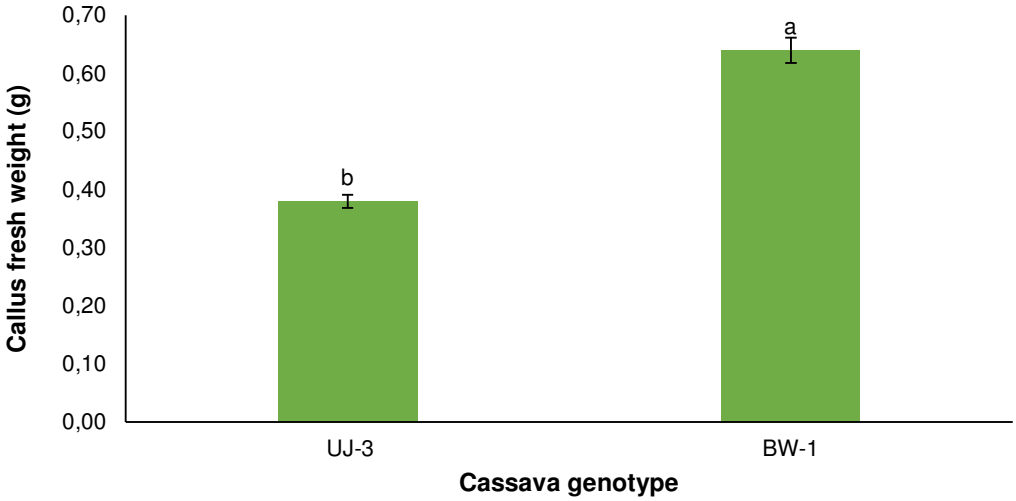

Figure 3. Fresh weight of callus derived from genotypes UJ-3 and BW-1 after induction on callus induction medium (4 weeks)
Each bar represents mean values (n=30) and error bar as standard error. Bars having same letters are not significantly different ($p \leq 0.05$) according to Least Significant Difference (LSD)

Somatic embryo germination and maturation

The embryogenic callus formed on CIM was subcultured on somatic embryogenesis development medium (SEDM) for germination and subsequent maturation of SEs. Two types of media were used, first medium was of same composition as CIM, and the second was with reduced concentration of picloram and NAA (SK1) in comparison to CIM. It was observed that the SEs differentiated on CIM were at the globular stage, and upon transferring on SEDM, it helped in maturation of SEs. The embryo formation started within 4 weeks from globular stage (Figure 1E, F) which continued to develop into elongated globular, heart and torpedo (Figure 1G), and cotyledon stages (Figure 1H). After four weeks on SEDM medium, the SEs were counted and it showed that their number varied between genotypes and PGR concentrations. The control medium (M0) failed to induce SEs in both genotypes. It was revealed that when the primary callus transferred from CIM to SK1 medium, differentiation of SEs was higher in SK1 as compared to CIM. The highest 46.56±36.86 SEs/explant with 67% frequency was obtained in BW-1 (Table 2); and they were further identified after 5 weeks using SEM which confirmed asynchronous growth of SEs as different stages were observed (Figure 4).

Plantlet regeneration and acclimatization

The callus with cotyledon stage SEs was transferred on plant regeneration medium (PRM) consisted of different concentrations of BA (0.2 or 0.4 mg/L). After 1-2 weeks in the PRM medium, the cotyledon embryos turned into green color, and multiple green shoot buds were formed (Figure 1I). Further, it was transferred on MS medium with or without 1% activated charcoal in order to develop shoots (Figure 1J). It was noted that within 3 weeks in this medium, green shoot were turned into normal plantlets (Figure 1K). The plantlets were then transferred to mini pot containing soil:cocopeat (1:1 w/w) and kept in culture room for four weeks (Figure 1L). Then they were transferred to greenhouse in small polybags for acclimatization, and gradual increase in plant height was observed after 2 months (Figure 1M) and 4 months (Figure 1N). The hardened plants showed morphological similarity with mother plants.

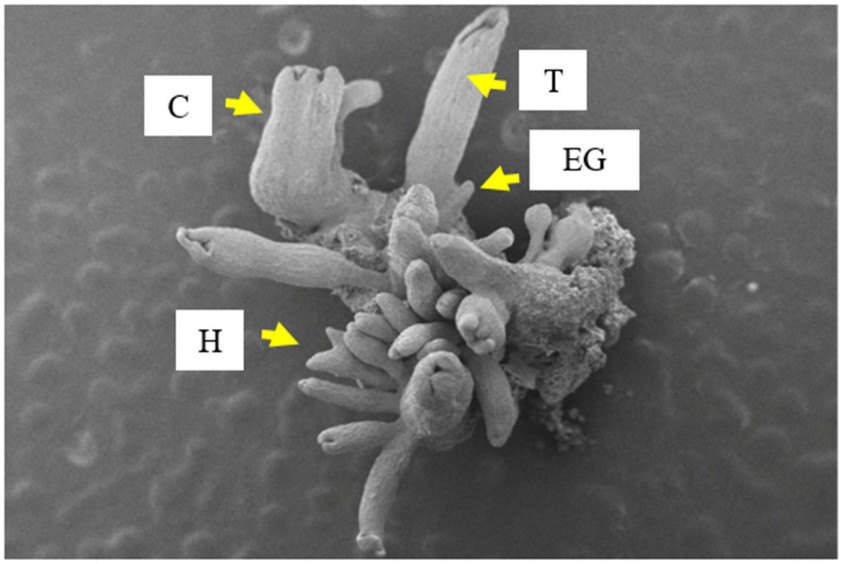

Figure 4. Distinguishing asynchronously growing SEs by scanning electron microscope. Different stages of SEs such as globular (G), elongated globular (EG), heart (H), torpedo (T) and cotyledon (C) stages (Scale bar: 200 µM, 50× magnification)

Table 2. Effect of different concentrations of picloram on somatic embryo induction and plantlet formation from two genotypes of cassava (8 weeks)

Genotype	SEDM	No. of SEs/explant*	Frequency (%)
UJ-3	M0	0 c	0 cde
	SK1	0 c	0 cde
BW-1	M0	0 c	0 cde
	SK1	0 c	0 cde
UJ-3	M1	2.22 ± 2.22 c	11.00 ± 0.11 bcde
	SK1	6.44 ± 3.50 c	33.00 ± 0.19 abc
BW-1	M1	0 c	0.00 ± 0.10 cde
	SK1	0 c	0.00 ± 0.10 cde
UJ-3	M2	7.11 ± 7.11 c	22.00 ± 0.22 bcde
	SK1	1.11 ± 1.11 c	11.00 ± 0.11 bcde
BW-1	M2	0 c	0.00 ± 0.20 cde
	SK1	46.56 ± 36.86 a	67.00 ± 0.00 a
UJ-3	M3	4.33 ± 4.00 c	22.00 ± 0.11 bcde
	SK1	11.89 ± 11.90 abc	44.00 ± 0.11 ab
BW-1	M3	0 c	0 cde
	SK1	6.00 ± 6.00 c	11.00 ± 0.11 bcde
UJ-3	M4	3.89 ± 2.21 c	22.00 ± 0.11 bcde
	SK1	9.33 ± 9.33 abc	11.00 ± 0.11 bcde
BW-1	M4	43.00 ± 40.69 ab	33.00 ± 0.19 abcd
	SK1	1.33 ± 0.89 c	33.00 ± 0.19 abcde

M0 = 0 mg/L picloram, M1 = 7.5 mg/L picloram, M2 = 10 mg/L picloram, M3 = 12.5 mg/L picloram, M4 = 15 mg/L picloram and SK1 = CuSO4 (4 µM) + picloram (6 mg/L) + NAA (0.5 mg/L). SEDM: Somatic Embryogenesis Development Medium

*Data represents Mean±SE (n=9). Means followed by same letters are not significantly different ($p \leq 0.05$) according to Least Significant Difference (LSD)

Discussion

Somatic embryogenesis is a process in which somatic cells develop to form true-to-type plants in a short time period. This pathway of regeneration is important for species like cassava as it can be used for genetic improvement through *Agrobacterium tumefaciens* as well as particle bombardment (Schopke *et al.*, 1997; Utsumi *et al.*, 2022). Moreover, the SEs can be also used for long-term storage through cryopreservation (Danso and Ford-Lloyd, 2004). Some factors involved in somatic embryogenesis are the origin of explant, *in vitro* culture condition, and culture medium (Nic-can *et al.*, 2015). In some plant species it is genotype independent e.g. *Camellia oleifera* (Zhang *et al.*, 2021), however somatic embryogenesis is affected by genotype and culture medium in cassava (Priadi and Sudarmonowati, 2006; Mongomake *et al.*, 2015; Ngugi *et al.*, 2015; Susanti *et al.*, 2017).

Picloram has been reported to induce callus in many studies (Priadi and Sudarmonowati, 2006; Opabode *et al.*, 2013; Mongomake *et al.*, 2015; Susanti *et al.*, 2017), which is in line with present investigation. It was noted that in both the varieties, primary callus developed from the cutting site as well as midrib of the explant, and it is in agreement with previous reports where primary callus originating from the cutting site or midrib eventually induce embryos (Anuradha *et al.*, 2015; Ngugi *et al.*, 2015). The observations also revealed that the colour of the callus was white in first week which later turned brown and ceased to grow. This decrease in growth is mainly due to the callus reached optimum cell division, its physiological degradation and/or lack of nutrients (Mahadi *et al.*, 2016). Previously, in Kibanda Meno Mkubwa cultivar of cassava it was reported that highest 88.97±1.73% frequency was obtained in presence of picloram (Marigi *et al.,* 2016). This frequency was lower as compared to present result achieved for cultivar UJ-3 (96.30±3.70%) and at par with BW-1 (88.89±6.42%). However, in control treatment containing NAA without picloram showed formation of non-embryogenic callus and roots in both the selected genotypes. It is well documented that use of varying concentrations of PGRs to induce callus in each genotype was affected by endogenous hormones (Saeedpour *et al.*, 2021). In addition, the augmentation of PGRs at different ratio regulates the type of organ differentiation (Bhojwani and Razdan, 1996), and similar to present result addition of NAA evoked rooting from leaf derived callus in *Portulaca quadrifida* (Pathak *et al.*, 2019). Present finding is also in accordance with earlier report in cassava where the embryogenesis failed to induce from leaf lobe explant in presence of NAA (Mongomake *et al.*, 2015).

Indirect somatic embryogenesis is a process in which somatic cells dedifferentiate and develop callus followed by formation of embryogenic clumps which later on differentiate SEs (Williams and Maheswaran, 1986; von Arnold *et al.*, 2002). In the present investigation when callus from both genotypes was transferred to SEDM, induction of SEs was observed in presence of picloram and NAA, however the response varied between them. Similarly, variation between two cultivars i.e. Ngan Mbada and Local Red of cassava has been well documented by Mongomake *et al.* (2015). They have reported that MS medium with picloram (50 µM) differentiated SEs with 40% response and average 90 no. of SEs in cultivar Ngan Mbada, whereas 33 µM 2,4-D has been proved suitable (40% of SE frequency and 60.5 average number of SE) for cultivar Local Red. Variation in response for cassava genotypes on induction of SEs has been also reported, in which Adira 4 differentiated embryo in presence of picloram, while genotypes Malang 6 and Sutra failed to form SEs (Susanti *et al.*, 2017). Whereas Opabode *et al.* (2013) reported that only three varieties out of eleven produced mature SEs on medium containing picloram (10 mg/L) for primary SE induction, which were further developed on medium supplemented with 0.1 mg/L BAP (Feitosa *et al.*, 2007). In the present study, optimum SEs were produced in presence of picloram and NAA which is in accordance with reports on *Eucalyptus globulus* and *E. saligna* × *E. maidenii* (Corredoira *et al.*, 2015). In both the selected genotypes it was recorded that medium augmented with reduced concentrations of picloram and NAA facilitated better SE development. This is in line with previous report on *E. globules* in which the proliferation of globular SEs increased by reducing NAA

level as well as in a medium devoid of PGRs (Pinto *et al.*, 2008). Likewise, better regeneration from the globular stage SE in Kenyan cassava was noted when decreased concentration or no PGRs were added in medium (Ngugi *et al.*, 2015). It can be concluded that PGRs plays an important role in the level of callus induction prior to embryogenesis, especially the auxins that affect dedifferentiation and induces cell division (Junairiah *et al.*, 2019). It was also noted that the growth of SEs was asynchronous in both UJ-3 and BW-1 genotypes, this is in corroboration with earlier reports on *E. globules* (Corredoira *et al.*, 2015) and *Leptadenia reticulata* (Patel *et al.*, 2021). It is possibly caused by an imbalance in the absorption of nutrients from the media into plant tissues (Hapsoro *et al.*, 2020).

The successful conversion of SEs into plantlet is a critical step as mature SEs are known to accumulate a sufficient amount of storage proteins and develop into normal plants (Rai *et al.*, 2008). It is well documented that this stage requires different PGRs as compared to germination medium (Pasternak *et al.*, 2002). In present investigation it was observed that conversion of SEs into shoots took place in presence of BA, and this in corroboration with results on giant bamboo (*Dendrocalamus giganteus*) (Ornellas *et al.*, 2022). Likewise, pivotal role of BA in SE development and maturation has been recorded in conversion of *L. reticulata* (Martin, 2004; Patel *et al.*, 2021) and *Eclipta alba* (Salma *et al.*, 2019) SEs. However, present investigation also revealed that some abnormal plantlets were formed along with normal plantlets. This might be due to long-term exposure to explant to a high level of auxin concentration which leads to induce somaclonal variation and this condition is known to affect the development of SEs into plantlets (Ngugi *et al.*, 2015).

Conclusions

In conclusion, a protocol for somatic embryogenesis from leaf lobe explants has been developed for two economically important Indonesian cassava genotypes UJ-3 and BW-1 for the first time. This protocol can be useful for mass-propagation which ultimately contributes to mitigate the exploitation of these genotypes from wild. The result will be also beneficial for seedling production and genetic transformation in these species which will improve the plant quality and productivity.

Authors' Contributions

Conceptualization: FY; Project administration: FY, SDU; Methodology: FY, SDU, TA; Data Analysis: FY, TA, AP; Literature review, writing and editing the manuscript: FY, AP; Funding acquisition: FY, SDU.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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