



HORMONAL REGULATION OF ROOT MORPHOGENESIS IN CALLUS CULTURE OF COWPEA (*Vigna unguiculata* L. WALP)

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ABSTRACT

Morphogenesis in high plants is controlled by manipulating the balance of auxins and cytokinins. In cowpea however, morphogenesis is not fully understood and very difficult to regulate. This study established a routine system for callus induction and root regeneration from primary leaves of cowpea (*Vigna unguiculata* L. Walp). Embryonic axes were used to establish seedling under in vitro condition and primary leaves from the in vitro seedlings were used as explants for the establishment of callus culture using different concentrations of 2, 4-dichlorophenoxyacetic acid (2, 4-D). To regenerate root, calli were subcultured on hormone free media or media fortified with 2.22 μ M N⁶-Benzylaminopurine (BAP). Callus induction was efficient (85.03%) on Murashige and Skoog (MS) basal salts with B5 vitamins (MSB5) fortified with 6.78 μ M 2, 4-Dichlorophenoxyacetic acid (2, 4-D) and induction frequency gradually decreases with reduction in the concentration of 2, 4-D. When calli were subcultured on MSB5 in the presence of N⁶-Benzylaminopurine (BAP), roots developed in 85% of the calli, with mean number of roots per callus ranging from 1 – 7. The presence of BAP was essential for roots development, as calli subcultured on hormone free media failed to develop roots, indicating the significance of hormonal balance in plant morphogenesis. The use of callus culture as experimental materials could help in understanding the processes of shoots and roots morphogenesis in cowpea. Root cultures could also be used in the production of secondary metabolites of pharmaceutical and other industrial values from genetically engineered callus.

Keywords: Root Morphogenesis, Callus culture, Plant hormones, Cowpea

INTRODUCTION

Cowpea (*Vigna unguiculata* L. Walp) is an important source of dietary protein in sub-Saharan Africa. The dry grains contain 23-32% protein and other important food components such as carbohydrate, lipid, fibre, minerals and vitamins (Nielson et al., 1993). About 7.56 million metric tons of cowpea is produced annually over an estimated area of 12.76 million hectares, with over 70% of the production coming from sub-Saharan Africa (IITA, 2007) (www.iita.org/cms/detail/cowpea_project_details).

The major problems to cowpea production in sub-Saharan Africa are insect pests and diseases which reduce the overall grain yield to 0.495kg/ha (FAO, 2012).

Development of cowpea varieties with resistance to major pests and diseases is impeded by the absence of resistance genes in the genome of cultivated cowpea varieties. Genetic engineering is a suitable avenue through which genes of interest could be introduced

into the genome of cowpea. For successful genetic improvement of cowpea, a reproducible in vitro regeneration protocol is required. A number of in vitro regeneration systems via organogenesis have been established for cowpea (Sani et al., 2015).

However, Cowpea appears to be recalcitrant for in vitro manipulations, especially via de novo regeneration. Due to difficulty in regeneration from callus culture, studies that involve in vitro culture of cowpea are difficult to carry out. In the last four decades, several investigations have attempted to develop callus base regeneration system in cowpea. However, there are complications on the influence of exogenous hormones on morphogenesis from callus culture of cowpea (Park et al., 2002). Earlier reports in cowpea (Nemeth, 1979; Hill et al., 1989; Amitha and Reddy, 1996) demonstrated that both cytokinins and auxins are required for morphogenesis in callus culture of cowpea. However, Anand et al. (2000) and Ramakrishna et al. (2005) reported embryogenesis

from callus culture of cowpea in the presence of auxin (2, 4-D) alone. Further researches remain to be conducted to ascertain the actual role of auxins and cytokinins on morphogenesis in callus culture of cowpea. This work examined the role of BAP and 2, 4-D on roots morphogenesis in callus culture of cowpea.

MATERIALS AND METHODS

Seeds of four cowpea varieties (SAMPEA 7, SAMPEA 9, SAMPEA 10 and SAMPEA 12) were obtained from breeding unit of Institute for Agricultural Research (IAR) Samaru, Zaria, Nigeria. The seeds were washed three times with liquid soap under running tap water and rinsed with distilled water. The seeds were then surface sterilized by initial dipping in 70% ethanol for two minutes and subsequent immersion in 2% sodium hypochlorite for 20 minutes. The seeds were then rinsed three times in sterile distilled water. Under aseptic condition, embryonic axes were excised from the seeds and cultured on MSB5 media; MS basal salts (Murashige and Skoog, 1962) and B5 vitamins (Gamborg et al., 1968). Embryos were allowed for two weeks to germinate and primary leaves of the *in vitro* plants were used as explants for callus induction. Primary leaves from embryonic axis derived 14-days-old in

in vitro seedlings were used as explant for callus induction. Leaves were excised using sterile forceps and surgical blades in the Laminar flow hood and dissected along their midribs into two. Leaf sections were placed on callus induction media (CIM) with abaxial parts in contact with the media. Eight leaf sections were cultured in a petri dish containing 30ml of autoclaved callus induction media (CIM) consisting of MSB5 fortified with 2.26 μ M, 4.52 μ M or 6.78 μ M 2,4-Dichlorophenoxyacetic acid (2,4-D) and five petri dishes were used for each treatment in a completely randomized design (CRD). Cultures were kept in the dark for three weeks. After three weeks of culture in the dark, callus induction frequency was recorded. To estimate callus growth 2g of compact pale green callus were excised and culture on fresh media containing the different concentrations of 2, 4-D. Callus proliferation was estimated by subtracting the initial weight from the final weight after three weeks of incubation in the dark. To induce root organogenesis, rapidly growing pale green embryogenic calli were selected and cultured on MSB5 with or without 2.22 μ M BAP. Cultures were kept under 16 hour photoperiod and root development was assessed. Roots regeneration frequency was determined by counting the number roots in each callus.

$$\text{Percentage Callus Formation} = \frac{\text{Number of leaf sections forming callus}}{\text{Total number of leaf sections cultured}} \times 100$$

Data collected were subjected to analysis of variance (ANOVA) and means were separated using Fisher's Least Significant Difference (LSD) at 5% significance level ($P \leq 0.05$) (R version 3.4.1, 2017). In case of percentage callus induction, data was transformed using arcsine $\theta = \sin^{-1}(\sqrt{P})$ before analysis and were converted back to percentages for presentation.

RESULTS AND DISCUSSION

Successful callus induction was achieved from primary leaves of *in vitro* seedling of cowpea. Leaf sections expended after one week of culture and callus formation was observed from the cut edges which gradually cover the whole explant. While no callus formation was observed on media without 2, 4-D, efficient callogenesis was recorded in the presence of 2, 4-D indicating the significance of the auxin in callus induction. In cowpea and other members of the *Vigna* species, 2, 4-D has been reported to be the most suitable hormone for callus induction (Park et al., 2002). This is because of its ability to revert cells in the explant to dedifferentiated state and begin to divide

rapidly (George et al., 2008). In addition to auxin wounding has been reported to promote callus formation in many plant species (Ikeuchi et al., 2013). This could be the reason why callus was first observed in the cut edges and gradually spread covering the entire explant.

The frequency of callus induction significantly increased with increase in 2, 4-D concentration in all the cowpea genotypes (Figure 1). The highest percentage callus induction (85.03%) was observed on media supplemented with 6.78 μ M 2, 4-D and percentage callus induction significantly ($P \leq 0.05$) decreases to 62.22% in media fortified with 2.26 μ M 2, 4-D. Similar observation was reported by Anand et al, (2000) and Ramakrishna et al, (2005) who observed efficient callus induction and proliferation from leaf explants of cowpea in the presence of different concentrations of 2, 4-D.

Three types of calli were observed; compact cream callus, whitish friable callus and brownish loose and watery callus (Plate 1 A & B). Callus quality (colour and texture) was significantly influenced by the concentrations of 2, 4-D. The lower concentration

(2.26 μ M) produced compact cream callus, and when the concentration of 2, 4-D was increased to 4.52 or 6.78 μ M a mixture of compact cream, friable white and soft brownish calli was observed. This finding is in line with report of Choudhary et al. (2009) who observed significant changes in colour and texture of callus with increased in 2, 4-D concentration in callus culture of Mung bean.

Compact cream callus was selected and cultured on media containing different concentrations of 2, 4-D for assessment of callus proliferation. Callus proliferation was however not affected by the concentration of 2, 4-D after three weeks of culture in the dark. When these calli were transferred and kept growing on hormone free media or media supplemented with 2.22 μ M BAP for root organogenesis, root development was not observed on hormone free media, but, adventitious roots formation was observed on media fortified with BAP. Earlier reports in *Bupleurum falcatum* and *Lactuca sativa* showed that adventitious root primordia in callus cultured on medium containing 2,4-D actively differentiated but the primordium did not protrude from the callus in the same medium (Bae et al., 1994; Kang et al., 1995). However, after the transfer of callus to auxin-free medium, the protrusion of root primordium from the callus occurred actively and the primordium grew, indicating that 2, 4-D stimulates the differentiation of root primordium in callus cultures but inhibits the growth of root primordium. In this study however, root formation was not observed on hormone free medium indicating that differentiation of adventitious roots in callus culture of *Vigna unguiculata* requires the presence of cytokinin. Earlier studies in cowpea (Amitha and Reddy, 1996; Soh et al., 1998) demonstrated that cytokinins were required for root development in callus culture of cowpea.

In addition, there were noticeable differences in the root frequency (number of roots per callus) among calli induced using different concentrations of 2, 4-D. The calli developed on media supplemented with lower 2, 4-D concentration (2.26 μ M) produced low number of roots per callus (1 to 2 roots per callus) and the number of roots significantly increased in calli induced with 4.52 or 6.78 μ M 2, 4-D (Figure 1C). Thus, it is deduced from this study that the concentration of 2, 4-D during the callus induction phase not only control the type of callus, but, also determines the morphogenic potential of the resulting callus.

Genotypic differences were observed in the frequency of callus induction and number of roots. SAMPEA 9

and SAMPEA 10 were better in callus formation concentration (Figure 1B), when compared with SAMPEA 7 and 12. Similar pattern was also observed in root frequency (number of roots per callus) (figure 1D). Our findings demonstrated that in cowpea, physiological conditions of the donor plant not only determine the callogenic potential of the explant, but, also control the morphogenic potential of the resulting callus. Similar observations were earlier reported reports in cowpea (Brar et al., 1999) and cereals crops (Souza Canada and Beck, 2013).

Strong interaction was also observed between cowpea genotype and 2, 4-D concentrations (Table 1). Increase in the 2, 4-D concentration from 2.26 to 4.52 or 6.78 μ M significantly increased the frequency of callus induction in SAMPEA 7, SAMPEA 9, and SAMPEA 10, resulting in more than 80% of the explants forming callus. This effect was observed in SAMPEA 12 only when 2, 4-D concentration was increased to 6.78 μ M. In SAMPEA 7, callus growth was significantly increased by all the 2, 4-D concentrations. On the other hand, only 6.78 μ M 2, 4-D influenced significant callus growth in SAMPEA 9 and SAMPEA 12. Highest number of roots per callus was observed in SAMPEA 9 and SAMPEA 10 in calli developed on media supplemented with 4.52 or 6.78 μ M 2, 4-D. This finding is line with work of Kucharska and Orlikowska (2009) and Bakshi et al. (2012) who also reported differences in regeneration competence of cowpea genotypes when exposed to high concentration of cytokinin during the shoots induction phase.

CONCLUSION

In conclusion, the 2, 4-D concentrations used in this study induced dedifferentiation and subsequent development of callus from leaf explant in cowpea. The nature and morphogenic potential of the resulting callus was determined by the concentration of 2, 4-D. It is also noteworthy that BAP is required to stimulate root formation in callus culture of cowpea, but considerable level of 2, 4-D is also required for development and growth of the adventitious roots. Thus, hormonal valance is critical in achieving morphogenesis in callus culture of cowpea.

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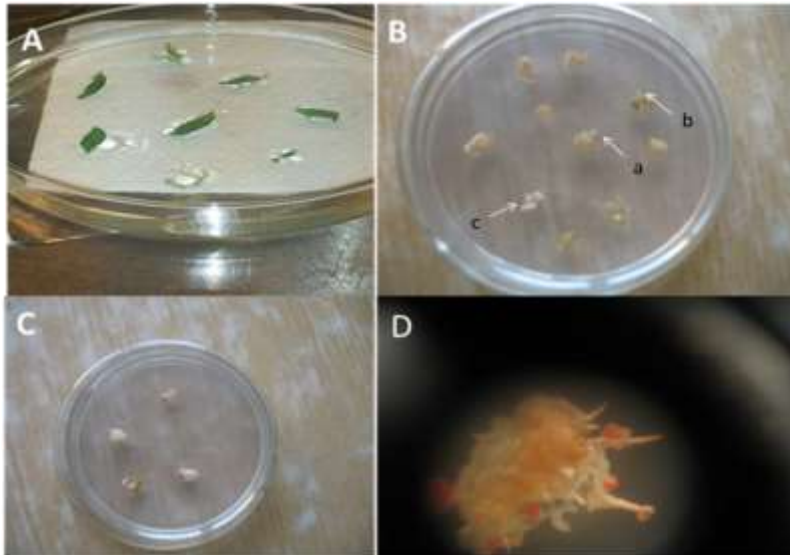


Plate I: Stages in roots regeneration by de novo organogenesis from primary leaves-derived callus in cowpea (*Vigna unguiculata* L. Walp). Explants were cultured on MSB5 supplemented with 30g/L sucrose and 100mg/L MES. (A) Longitudinally dissected primary leaves cultured on callus induction media (B) Development of callus 4 weeks after cultivation on MSB5 + 2, 4-D (a) pale green compact callus, (b) brownish loose callus and (c) White friable callus). (C) Callus proliferation 4 weeks after subculture on MSB5 + 2, 4-D. (D) Roots developed following callus subsequent culture on hormone free media and media fortified with 2.22µM BAP under 16 hours photoperiod.

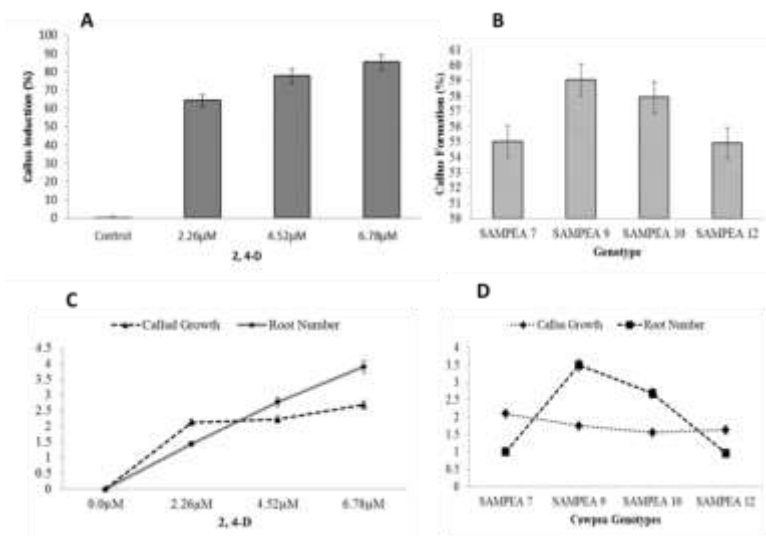


Figure 1: Effect of 2, 4-D and cowpea genotypes on frequency of callus formation callus growth and number of roots in cowpea. A - effect of 2, 4-D on callus formation, B - effect of genotype on callus formation, C - effect of 2, 4-D on callus growth and number of root, D -effect of genotype on callus growth and number of roots

Table 1: Influence of 2, 4-D on percentage callus formation and roots number in four different genotypes of cowpea.

Variety	2,4-D (μM)	Callus Induction (%) \pm SE	Callus Growth Means \pm SE	Number of Root Means \pm SE
SAMPEA 7	0.00	0.30 \pm 0.02 ^c	0.0 \pm 0.0 ^d	0.0 \pm 0.0 ^d
SAMPEA 7	2.26	58.6 \pm 4.7 ^d	2.6 \pm 0.2 ^{ab}	1.0 \pm 0.0 ^{cd}
SAMPEA 7	4.52	80.6 \pm 2.9 ^{ab}	2.8 \pm 0.2 ^{ab}	2.0 \pm 0.1 ^{bc}
SAMPEA 7	6.78	80.6 \pm 3.9 ^{ab}	3.0 \pm 0.1 ^a	2.0 \pm 0.2 ^{bc}
SAMPEA 9	0.00	0.10 \pm 0.01 ^c	0.0 \pm 0.0 ^d	0.0 \pm 0.0 ^d
SAMPEA 9	2.26	66.3 \pm 0.7 ^{cd}	2.3 \pm 0.2 ^{bc}	2.0 \pm 0.2 ^{bc}
SAMPEA 9	4.52	80.5 \pm 4.3 ^{ab}	2.0 \pm 0.1 ^c	6.0 \pm 0.3 ^a
SAMPEA 9	6.78	89.4 \pm 4.6 ^a	2.8 \pm 0.2 ^{ab}	7.0 \pm 0.5 ^a
SAMPEA 10	0.00	0.00 \pm 0.0 ^c	0.0 \pm 0.1 ^d	0.0 \pm 0.0 ^d
SAMPEA 10	2.26	67.6 \pm 3.0 ^{cd}	2.3 \pm 0.1 ^{bc}	2.0 \pm 0.1 ^{bc}
SAMPEA 10	4.52	87.8 \pm 3.6 ^a	1.8 \pm 0.2 ^c	4.0 \pm 0.3 ^b
SAMPEA 10	6.78	87.2 \pm 4.9 ^a	2.3 \pm 0.2 ^{bc}	6.0 \pm 0.5 ^a
SAMPEA 12	0.00	0.00 \pm 0.0 ^c	0.0 \pm 0.0 ^d	0.0 \pm 0.0 ^d
SAMPEA 12	2.26	64.4 \pm 0.6 ^{cd}	1.8 \pm 0.3 ^c	2.0 \pm 0.3 ^{bc}
SAMPEA 12	4.52	72.4 \pm 4.1 ^{bc}	2.0 \pm 0.1 ^c	1.0 \pm 0.1 ^{cd}
SAMPEA 12	6.78	82.9 \pm 0.5 ^{ab}	2.8 \pm 0.2 ^{ab}	1.0 \pm 0.0 ^{cd}

Means with same letter along the column are not significantly different at $P \leq 0.005$