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IN VITRO GERMINATION AND TISSUE CULTURE PROTOCOLS ESTERBLISHMENT OF COWPEA (VIGNA UNGUICULATA L. WALP) BORNO BROWN

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ABSTRACT

Cowpea faces several biotic and abiotic stresses for which conventional breeding alone may not provide ultimate solution.Hence, the present investigation was carried out to standardize in vitrogermination and tissue culture protocol. The germination of the cowpea (vigna unguiculata l. walp) Borno Brown, seeds were carried out on a water agar medium without any growth regulator, which germinated in 3-4 days. Mercuric chloride (0.1%) treatment for 1 min which immerse quickly in distilled water, followed by quick immersion in 70% ethyl alcohol for 1 min was found. Out of 200 hundred test tube 96.33% cotyledon were developed. Irrespective of growth regulators, maximum callus induction response was observed in stem explants (62.08%) followed by embryo axes and embryo axes with single cotyledon, with minimum callus induction in leaf disc. The explants shown highest callus induction response in MS medium supplemented with 1.0 mg/l IAA + 0.5 mg/l BAP. Maximum callus induction response in embryo axes explants (76.44%) on (MS + 1.0 mg/l IAA + 0.5 mg/l BAP). Number of days required for callus induction ranged from 12 to 14 days in cowpea and produced maximum weight of callus (64.74 mg) and it was greenish yellow and friable. Between the explants, mature embryo axes with single cotyledon (EAWSC) were found to be superior over embryo axes explants with shoot induction response (74.1%). Days to shooting ranged between 6 to 7 days. MS medium supplemented with 0.5 mg/l NAA + 0.5 mg/l kinetin gives maximum root induction response. The maximum per cent root response (63.44%) and rooted plants were acclimatized to potting mixture of soil, sand and vermiculite

INTRODUCTION

Cowpea is one of the most cultivated species in Legume family (**Jacobsen, 1991***)*.Cowpea (*Vignaunguiculata*) and Bambara nut (*Vignasubterranea*) are the two species domesticated inAfrica among the genus *Vigna*.(**Ba *et al*.,2004**). The cowpea (*Vigna unguiculata L. Walp*) initially found to exist in West Africa, mostly in the sub-humid savanna grasslands of Nigeria, and was domesticated about 4000 years ago from the wild progenitors *V.unguiculata*. (**Lush, 1981**).It is consumed in tropical and sub-tropical countries of Africa, America,and Asia. It is an essential source of dietary protein with both its dry grain and vegetative parts containing 23-32% protein and other vital food components such as fiber, lipid and vitamins **(Adeyemi*et al*., 2012and Gepts*etal*., 2008)**. About 80% and above production cowpea comes from West Africa with Nigeria, Niger, Chad and Burkina Faso accounting for 77% of the production (**FAOSTAT; 2012**). Due to its drought tolerance and ability to thrive well in low fertile soils, it is cultivated as a security crop by small scale farmers..It is now cultivated throughout sub-Saharan Africa, Southeast Asia, Latin America, and the United States and occupies an area of 10.5 million hectares with a world annual production of about 3.9 million tons (**FAOSTAT, 2006**).Cowpea requires lower water and nutrition compared to other legumes (**Da Costa and Lobata, 2011**).

The application of biotechnology tools to agriculture has allowed for the transformation of plants without the need for sexual compatibility between species, thus establishing the possibility of rapidly producing new crop varieties. Plants have been transformed successfully to improve their pest and disease resistance, herbicide tolerance, nutritional qualities, and stress tolerance (**Machuka*et ai*.,2002**).*In vitro* tissueculture is among the key tools of plant biotechnology that developed the nature of plant cells, a concept proposed by (**Haberlandt*et al*.,1902**).

Furthermore, variability is involved in almost every aspect of the regeneration systems explored, such as optimal basal salt compositions, explants tissues, plant growth regulators, sucrose levels, and variation in composition of gelling agents (agar or Gelrite) (**Pellegrineschi, 1997; Popelka*et al*., 2006; Aasim*et al*., 2009a**). Based on plant tissue culture using various explants is difficult and highly cultivar-dependent establishment of an efficient and reproducible transformation system (**Muthukumar*et al*., 1995; Anand*et al*., 2001; Choi *et al*., 2003; Chaudhury*et al*., 2007**)

Increases in light levels and improving the carbon dioxide availability, either through venting or artificial enrichment, make plant tissue to be grown photo-autotrophically in a vessel. Photoautotrophic micropropagation means micropropagation with no exogenous organic compound (i.e. sugar, vitamins etc) added to the growth medium. Improvement of photo-autotrophic micropropagation for the production of plantlets has shown that this method raises carbon dioxide and light levels and the lack of sucrose in the medium increases the growth of the plantlets (**Zobayed, *et al*., 2000**). In observation which shows that plants can grow rapidly in the absence of sugar if provided with good ventilation system coupled with high photosynthetic photon flux (PPF) of 100-150 μmol m -2 s - 1 and lesser percentage of relative humidity in the

culture vessel (**Chun and Kozai 2001**). In general, the culture medium plays a vital role in the ability of the explants to regenerate shoots, with the specific medium requirements being highly dependent on the plant species. (**Zaidi*et al*., 2005**). Nutrient medium pH ranges from 5.0 to 6.0 for suitable in vitro growth of explants. pH higher than 7.0 and lower than 4.5 generally stops growth and development.

Records of several efforts have been reported on tissue culture practices on cowpea. **(Kobuyama*et al*. 1992**) studies that the use of embryo culture to generate mature plants *in vitro* from hybridization of three lines of the garden bean (*Phaseolus vulgaris L.*). Many publications are available on the micropropagation of various *vigna* species. A protocol for the induction of multiple embryo shoot formation in axillary explants from zygotic embryos of *P. vulgaris* as well as for the regeneration of shoots to fertile plants was improved.The objective of this study work is to identification of suitable tomato variety for tissue culture,to evaluation of callus generation for the identified seeds using various combinations of auxin and cytokinin and to stablishment of multiple shoot development, rooting and hardening of tomato plants.

MATERIALS AND METHOD

Materials

Procurement and Sterilization of seeds

Viable seeds of cowpea (*Vigna unguiculata)* were obtained fresh from Department of agriculture, Ramat polytechnic Maiduguri, Nigeria. Seeds were surface sterilize withMercuric chloride (0.1%) treatment for 1 min which immerse quickly in distilled water, followed by quick immersion in 70% ethyl alcohol for 1 min was found to be most effective treatment to get maximum (87.50%) aseptic cultures, and the seeds would be rinse 3 times in sterile distilled water. The seeds would split open with embryo and attach to one cotyledon using sterile forceps and scalpel.

Nutrient medium

Murashige and Skoog (1962) medium, the most commonly used tissue culture medium has been used along with different concentrations of plant growth hormones.The basal medium was prepared. The pH of the medium was adjusted to 5.8 by using 1 N NaOH or HCl. Volume was finally adjusted and 10 g/l of agar was added to the medium and then dispensed into suitable container. Stock solution of auxins like Indole Acetic Acid (IAA), Naphthalene Acetic Acid (NAA); and cytokinins like 6 Benzyl Adenine Purine (BAP), Kinetin were prepared and added into basal medium depending upon the purpose of individual experiments.

Explants Preparation

Treated seeds were also inoculated on the water agar medium and allowed germination for 3-4 days in the absence of light.The germinated seedlings were brought out to light for isolating explants like leaf disc. The embryo axes, embryo axes with single cotyledon and leaf disc explants were separated by using sterilized stainless steel knife and inoculated on culture medium containing growth substances for callus induction and *in vitro* regeneration.

Transfer of explants to callusing medium

Embryo axes, embryo axes with single cotyledon, stem and leaf disc were used for callus induction. As many as 3 to 4 explants (embryo axes, embryo axes with single cotyledon and leaf disc)

were inoculated in test tubes containing 15ml of culture medium, conical flasksand Petri-plates containing 20 to 22 ml of culture medium. During theinoculation of explants, all the surgical instruments were first dipped inrectified spirit, flamed on spirit lamp and cooled before use.To ensure close contact with the medium, the explants were pressedgently. Culture bottles/test tubes/ conical flasks were covered with suitablecaps or plugs of non-absorbent cotton to avoid contamination. Theseexplants were placed in light with controlled humidity and temperature for 2weeks for callus induction. The opened leaf should be placed into the callusing medium and the callus appearance should be observed for 15-30 days. After 3-4 weeks of formation of calli, it has been sub cultured on another fresh medium containing various combinations of auxin and cytokinin and shifted into light condition once rooting is observed. That was done to expose the calli to more IAA concentration and for further growth and multiplication of the calli.After 15 days, from last sub culturing of calli, next sub culturing was done for regeneration with different concentrations of cytokinins.

After development of multiple shoots, the well-grown elongated shoots were transferred to the rooting media containing different concentration of auxin and cytokinins the plantlets were separated from each other and transferred on the rooting media for further root development.Root induction response (%), days to rooting, number of roots were observed. While transferring rooted shoots from culture, roots were gently washed with distilled water to remove the medium attached to them, and numbers of plants survived with normal growth.

Result and Discussion

Present studies carried out to*invitro*germination and tissue culture protocol through somatic embryogenesis and direct organogenesis of cowpea in the laboratory of Biotechnology department, and the results obtained are presented in this chapter.

4.1Germination of cowpea seeds

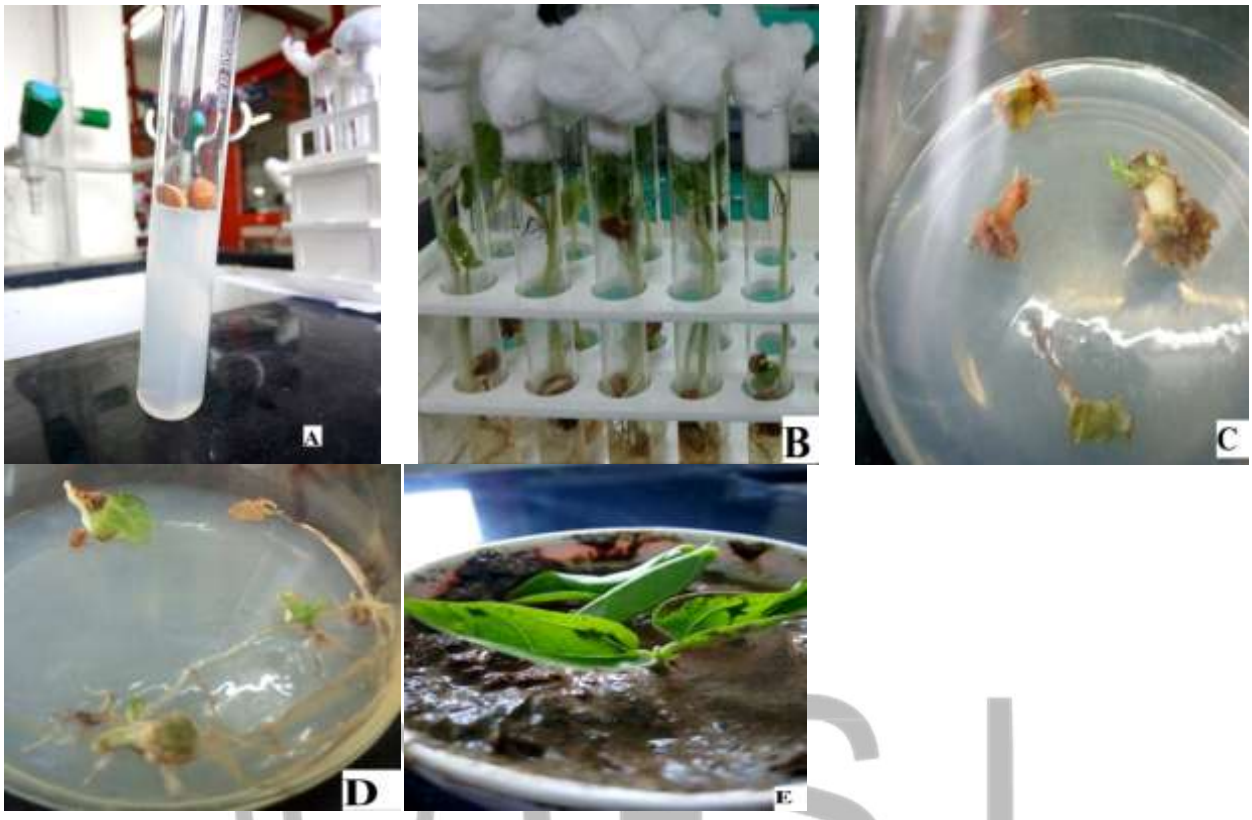

*Figure (***A***) show the inoculation of cowpea in water agar medium in 200 different test tubes for germination, which were kept in the absence of sun light (dark condition) at room temperature.(***B***)Explants regeneration observed in the somatic embryo after exposure to the sun light.(***C***) Shoot explants response in the new MS medium supplemented with IAA and BAP.(***D***)Rooting of shoots response on MS medium supplemented with NAA and kinetin.***(E)*Rooted plants were transferred to the potting mixtures containing soil, sandand vermiculite.*

After 3 days of incubation 96.33% of the sample seeds were successfully germinated by cotyledon opening and produce somatic embryo, which were then brought to sun light exposure.
Figure B 2 days after exposure of somatic embryo to the sun light, 98.3% of them were developed explants with green leaf, stem, and roots. The leaf disc and stem explants were transferred to MS medium for the callusing induction as shown in fig B.
Figure 3:
Note: The explants initiate callusing induction after 12-14 days of inoculating and it turned complete brown color.Among the four explants, significantly highest response for callus induction was observed in stems (62.08%), followed by embryo axes (60.33%), embryo axes with single cotyledon (57.77%) and leaf disc (54.22%) . Mean callus induction response ranged from 49.56 per cent to 76.44 per cent in all explants. The maximum weight of callus was 64.74 mg observed. The calluses were then transferred to new MS medium containing NAA and

kinetin for shoot regeneration response. After 1 week regeneration of shoot explants were initiated.

In figure 4 the multiple Shoots explants induction were observed after 6-7 days of transfer of the callus into the new MS medium containing IAA and BAP. The result shows 74.1% response of shoot bud with maximum number of 3.30 shoot buds per explants. The shoot explants were again sub-cultured into a new MS medium which supplemented with NAA and kinetin for the elongation of shoot explants and initiation of roots. Cytokinin commonly stimulates shoot proliferation and inhibits their elongation, due to its high cytokinins activity. Generally reduction in cytokinin levels in regeneration medium favours proper elongation of shoot buds.

The maximum per cent root response (63.44%) in the MS medium containing NAA and kinetin was observed and the maximum number of roots observed was (6.16) with minimum number of roots was (3.11). The early rooting induction was observed (9.77 days) and the late root induction observed is (14.5 days).Rooted plants were transferred to the potting mixtures containing soil, sand and vermiculite. The number of plantlets survived 60 per cent in potting mixture was observed.

CONCLUSION

These studies however indicated that in cowpea, *in vitro* regeneration responses requiring the optimization of tissue culture. Furthermore variability involved in every aspect of the regeneration systems explored such as optimal explants tissues, basal media combinations and plant growth regulators.

The presence of variability for callus induction and direct regeneration offers a great scope for selecting most suitable genotypes for creating somaclonal variation and production of insect pest resistant plants through genetic engineering.

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