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SOMATIC EMBRYOGENESIS IN LOCAL COWPEA (*Vigna unguiculata* L. (WALP) VARIETIES

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ABSTRACT

Organogenesis *in vitro* was evaluated in explants derived from shoot apices of two local cowpea varieties cultured on Murashige and Skoog (MS) basal medium. To evaluate the response of the cowpea genotypes to *in vitro* regeneration, seedlings were drived from zygotic embryos excised from surface sterilized seeds after two weeks of culture on MS basal medium + vitamins supplement with 30% sucrose. To regenerate shoots, apices of the *In Vitro* seedlings with average length of 1cm were excised and cultured on MS supplemented with 0.5, 1.0 or 1.5mg/L 6-Benzylamino purine (BAP) with or without 0.1mg/L α-naphthaleneacetic acid (NAA) for six weeks. Variation was observed in response of the shoot apices from different cowpea cultivars to *in vitro* regeneration of shoots. The highest shoot number was recorded with Danilla and shoot length was highest in Kanannado, indicating that Danilla responded well to *in vitro* regeneration. While there were significant differences between these concentrations and other concentrations used in this study on the number of shoots produced, statistically there was no difference between the traetments tested on the *in vitro* shoot length recorded in this study.Although shoots were succesfully regenerated in this study rooting was not achieved as the explants gradually transformed into calli.

Keywords: Organogenesis, Explant, Somatic Embryogenesis

INTRODUCTION

Cowpea (*Vigna unguiculata) L. Walp* is a staple food crop of significance worldwide. Major diversity in cowpea is found in Asia and Africa, but the precise origin of cowpea has been a matter of speculation and discussion for many (Monti, 1997). Early observations showed that cowpea genotypes in Asia is very diverse and morphologically different from those in Africa. Therefore, both Asia and Africa were thought to be independent centers of origin of cowpea (Christou, 1992). Cowpea plays a critical role in the lives of millions of people in Africa and other parts of the developing world, where it's a major source of dietary. Singh, 2000.

However, in the absence of wild cowpeas in Asia as possible progenitors, Asian center of origin has recently been questioned. All the current evidence suggests that cowpea originated in southern Africa although it is difficult to ascertain where in Africa the crop was first domesticated. Several centers of domestication have been suggested. These include Ethiopia, Central Africa, South Africa and West Africa. Based on the distribution of diverse wild cowpea in Eastern Africa stretching from Ethiopia to Southern Africa, the working group meeting of the International Board for Plant Genetics Resources on *Vigna* held in New Delhi in 1981 recommend as a priority, collection of both wild and cultivated forms of cowpea in Southern Africa, Zimbabwe, Transvaal and Natal. East and Southern Africa are considered as the primary regions of diversity and West and Central Africa to be the secondary centers of diversity. India in particular and Asia in general have been proposed to be the third centers of diversity.

Recent investigations by the International Institute of Tropical Agriculture (IITA) in collaboration with Institute of Germoplasmo (CNR) Bari, Italy strongly indicate that the region encompassing Namibia, Botswana, Zambia, Zimbabwe, Mozambique, Swaziland and South Africa have the highest genetic diversity in respect of primitive wild forms of cowpea. Some very primitive species were observed in the Transvaal, Cape Town and Swaziland. Based on this, it has been suggested that southern Africa may be the center of origin of cowpea and from there to Asia and West Africa. Cowpea was known in India before Christ and it has Sanskrit name in early treatise dating back to 150BC. Cowpea must have moved from east Africa to Asia more than 2000 years ago where human selection led to modified forms of cowpea different from Africa. It has been suggested that cowpea probably moved from Eastern Africa to India before 150BC to West Asia and Europe about 300 BC and to Americas in 1500 AD. Since western Asia and Europe do not have desired climatic conditions for cowpea cultivation, not much variability and selection occurred as it happened in South Asia and South East Asia, where small seeded and vegetable cowpea were selected. Probably, the wild cowpeas with very small seed were distributed by birds in east and west Africa much before Christian era and therefore the presence there of great diversity and secondary wild forms. Selection for larger seeds and better growth habits from natural variants in wild cowpeas by humans must have led to diverse cultigroups and their domestication in Asia and in Africa.

It is believed that cowpea reached south-western Asia about 2300BC and was introduced to southern Europe early enough for the Greeks and Romans to grow it under the names *Phaseolus* and *Phaselus* (Allavena and Rosseti ,1986). Its supposed that cowpea reached India more than 200 thousand years ago from east Africa through the Sabenia lane along with other crops like sorghum, finger millets and bulrush – millet. Cowpea was introduced to the new world in the late seventieth century by the Spanish with more cultivars transported there from West Africa through the slave trade (Chourera *et al.*, 1995).

Cowpea reached the United States in the early nineteenth century. The actual region of domestication is also speculative, West Africa is believed to be the region where there is maximum diversity of cultivated cowpea and this

encompasses the savanna region of Nigeria, Southern Niger, Burkina Faso, Northern Benin, Togo and the North western part of Cameroon (Monti, 1997). Cowpea has considerable adaptation in high temperature and drought compared to other species. Hall *et al*, 2002.

MATERIALS AND METHODS

Plant Materials

Two (2) varieties of local cowpea comprising of(Kanannado and Danila obtained from Ajingi local government local community Kano, were used as donor materials for this study. Kano is located between the latitude 12.00°N and longitude 8.50°E with tropical type of climate they characterized by two distinct seasons, wet and dry

Experimental site for the *In-vitro* Study

The study was carried out in the Plant Biotechnology laboratory, of Jigawa Research Institute, Kazaure, Jigawa State, Nigeria.

Seed Sterilization

The seeds were washed four (4) times in running tap water, followed by immersion in 70% ethanol for two (2) minutes and sterilized by immersion in 20% commercial bleach (5% sodium hypochlorite). This was followed by addition of few drops of tween 20 with rigorous shaking at three (3) minute interval. Tween 20 ensures contact between the hypochlorite and seed. Finally, the seeds were rinsed three (3) times in double distilled water.

Medium and Culture Conditions

The medium used in this study was Murashige and Skoog (MS) (Murashige and Skoog, 1962) basal medium, which consist of macro and micro-salt and vitamins. The medium was supplemented with 30% sucrose, pH was adjusted to 5.8 with 1M KOH and solidified with 8% agar before autoclaving for 15 minutes at 121ºC. All cultures were incubated at 27 ± 2ºC.

Composition of Murashige and Skoog (1962) Basal Medium

Macro – elements	**Concentration (mg/l/L)**
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
KH_2PO_4	170
$Mg/lSO_4.7H_2O$	370
Micro – elements	**Concentration (mg/l/L)**
H_3BO_3	6.20
$NaEDTA.2H_2O$	37.30
$MnSO_4.4H_2O$	22.30
$FeSO_4.7H_2O$	27.8
$ZnSO_4.7H_2O$	8.6
Kl	0.83
$Na_2MgO_2.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CaCl_2.6H_2O$	0.025
Vitamins	**Concentration (mg/l/l)**
Myoinositol	100
Nicotinic acid	0.5
Pyridoxine-HCl	0.5
Thiamine-HCl	0.5
Glycine	2.0
Carbon Source	**Concentration (mg/l/l)**
Sucrose	30,000

Embryo Culture

Complete embryos were excised by the use of forceps and surgical blade. The cotyledons were first opened, then the embryo were excised and placed in culture bottles containing 35ml of the medium and the bottles were sealed with paraffin film. The culture bottles were then incubated in a growth chamber at 27 ± 2ºC, under 18 hour photoperiod for fourteen (14) days.

Shoot apices with average length of 1cm were excised from the *in vitro* seedlings and used as explants for the *in vitro* regeneration of multiple shoots..

Plant Regeneration

Five (5) apices were placed in shoot initiating medium contained in the culture tubes. Shoot initiating medium consisted of basal medium supplemented with different concentrations of BAP (0.5, 0.1 or 1.5mg/l/L) with or without NAA (0.1 mg/l/l). Eight (8) tubes were used for each treatment and the experiment was laid in a completely randomized design with three (3) replications. Culture tubes were incubated for six (6) weeks at 25 ± 2ºC under 16hrs photoperiod. Number of explants growing multiple shoot, number of shoots per explant and mean length of shoots were recorded for each treatment.

RESULTS

In each traetment the number of apices that responded to *in vitro* regeneration by producing single or multiple shoots were recorded (Figure 1). The result showed that, Danilla was the highest with 90% of the explants producing single or multiple shoots when subjected to *in vitro* regeneration in the presence of BAP with without NAA.. The least among the genotypes was Kanannado with 75% response.

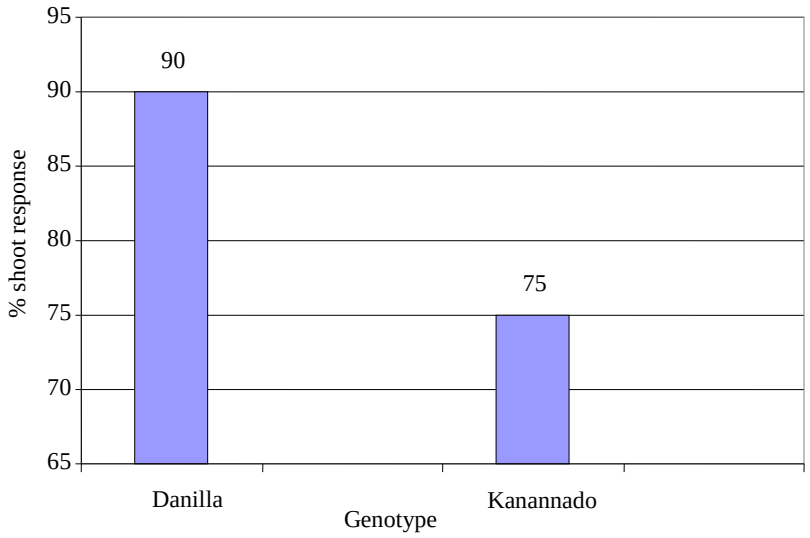

Figure 1: Shoots regeneration response (%) to *In Vitro* regeneration

The effect of the treatments on the percentage of explants responding to regeneration was also recorded (Figure 2). The result showed that supplementing the media with 1.5mg/LBAP with 0.1mg/L NAA recorded the highest response (100%) and was closely followed by 1.5mg/L BAP, and 1.0mg/L BAP with and without NAA.However, the lowest response was recorded in media supplemented with 0.1mg/L NAA alone and the control.

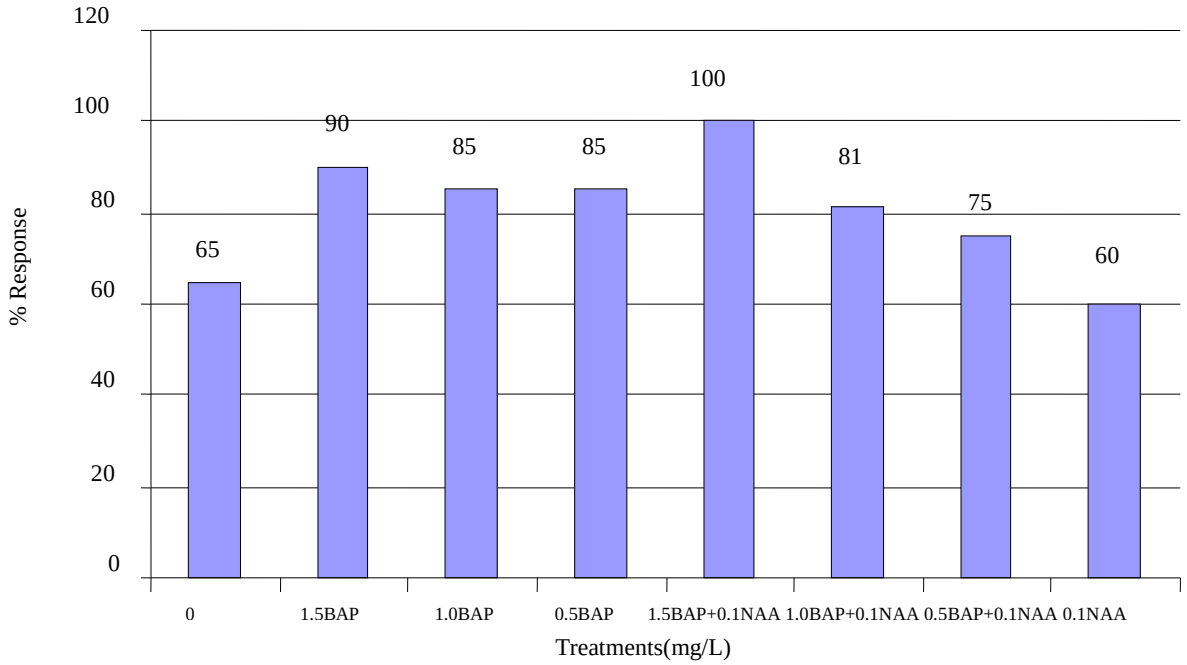

Figure 2: Percentage regeneration of the Cowpea Genotypes to *in vitro* regeneration under different treatments.

The effect of hormone supplements on the number of shoots is illustrated in Figure 3. Highest mean shoot number was recorded in media supplemented with 1.5mg/l BAP + 1.0mg/l NAA with mean shoot number of 2.89a. This was significantly different from 1.5mg/l BAP alone with mean shoot number 2.51b. Supplementing the media with 1.0mg/l BAP $\pm$ 1.0mg/l NAA produced a mean shoot number of 2.03C and is significantly different ($P< 0.0001$) from 1.0mg/l BAP alone (1.58d) and 0.5mg/l BAP alone (1.65d) and with 0.1mg/L NAA (1.71d). The least response in relation to the number of shoots was obtained in media supplemented with 0.1mg/l NAA (0.97e). This was not statistically different from the control with mean shoot number of 1.15e.

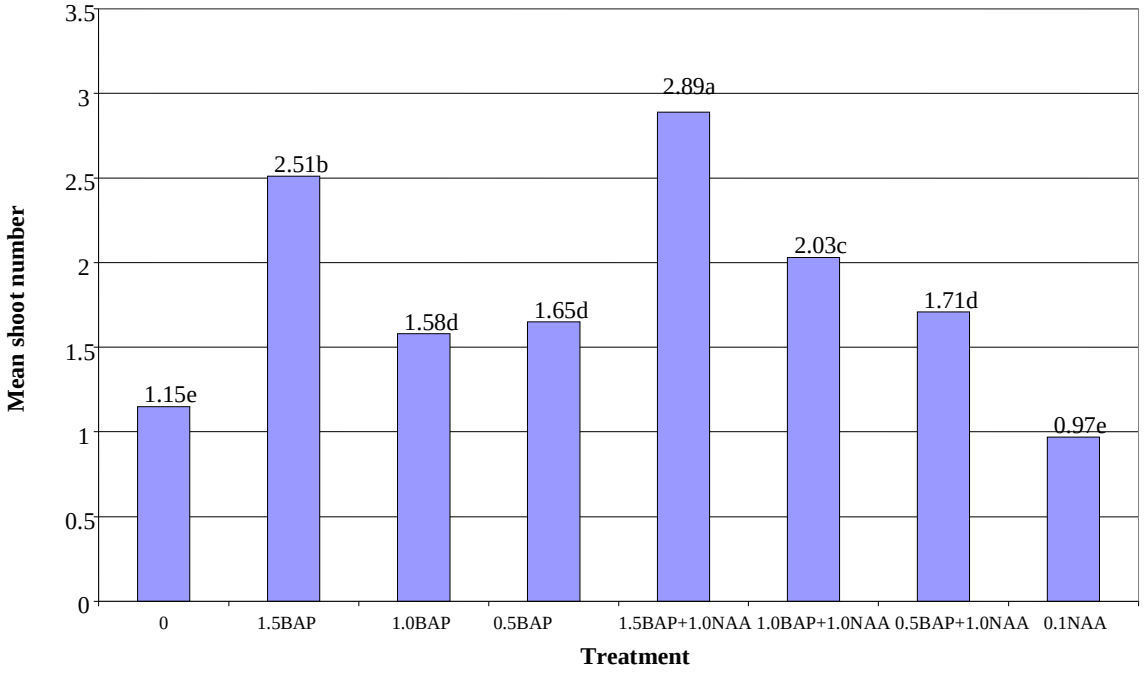

Figure 3: Effect of Hormone Supplementation (BAP ± NAA) on *In Vitro* Shoot Number in cowpea explants on MS media

The response of cowpea genotypes in term of shoot length is also evaluated Fig.4. The genotype that recorded the highest mean shoot length was Kanannado (2.49a) followed by Dan'ila (2.47a) and IT04K -332-1 with a mean shoot length of (2.43a). The response of these genotype was significantly different from IT99K-573-2-1 with a mean shoot length of (2.17b).

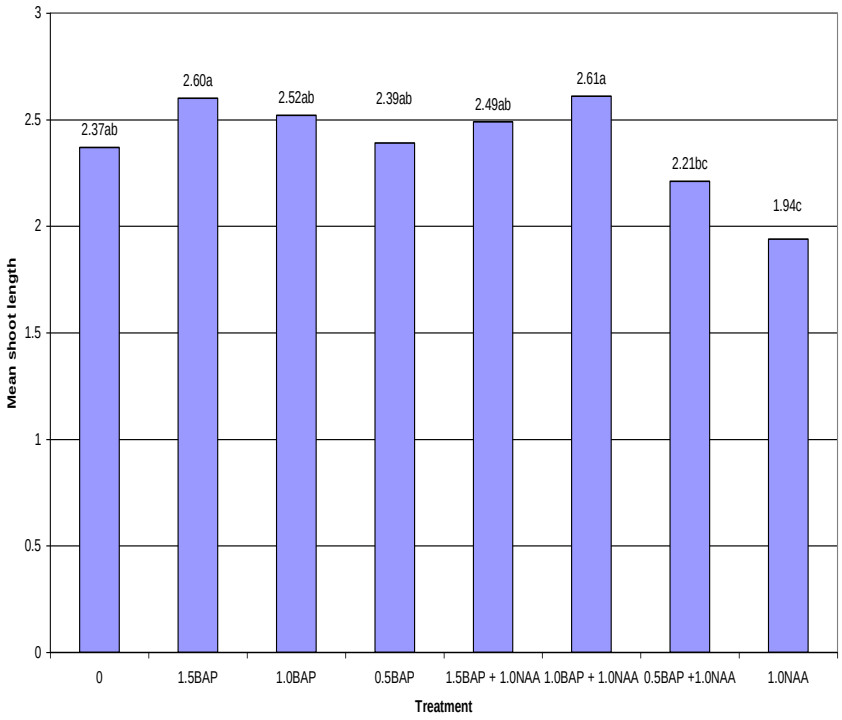

Figure 4: Effect of Different Concentration of BAP ± NAA on *In vitro* shoot length in cowpea explants.

Plate 1: Regenerated multiple shoots on MS supplemented with 1.5mg/L +0.1mg/L NAA

DISCUSSION

Direct multiple shoot induction is the useful means of production of plantlet with a lower risk of genetic instability than by the other regeneration routes such as somatic embryogenesis (Rao and Lee, 1986). In the present study efforts have been intensified to initiate *Vigna unguiculata*direct multiple shoots from shoot apices excised from 14 days-old *in vitro* raised seedlings. After ten (10) days in induction medium containing Benzylaminopurine (BAP) with or without α-Naphthalene acetic acid (NAA), the explants showed swelling at the cut (basal) edges from which adventitious buds developed after 3 weeks in culture. However in induction medium without growth regulators (control) explants did not show any swelling at the cut edges. About 90% regeneration rate of the explant was obtained in all media tested. This is true with Machuka *et al,* 2002 where media were supplemented with BAP & NAA, multiple shoot production in cowpea was possible.

The frequency of *in vitro* multiple shoots formation was significantly affected by the concentration of plant growth regulators. *Vigna unguiculata* shoot apices produced multiple shoots when cultured on MS medium supplied withBAP in combination with NAA. However, the mean number of shoots per explant varied significantly with varying concentrations of BAP in themedium. Among the various concentrations of BAP tested, 1.5mg/L + 0.1mg/L NAA(2.89) and without NAA (2.51) resulted in formation of the highestnumber of shoots after six weeks of culture.These results indicate that *Vigna unguicula*shoot apices have a considerable regenerative capacity.Results of this work are in line with report of Kononowiez *et al* (1997) and Monti *et al* (1997) which reported that various genotypes, including CB5, TARS36, SUV-2, 1137, 275, Tn88-63 B301, TVU 9062, Vita 3, Vita 4 and 58-57 were regenerated from shoot meristem culture on MS supplemented with BAP.*In vitro* regeneration of cowpea via organogenesis has also been reported in several genotypes of cowpea (Machuka *et al*., 2002). However when 0.1mg/L NAA alone was applied to the media the proliferation of multiple shoots significantly reduced (0.9) indicating that exogenous NAA was not essential to initiate shoot bud formation. In consistence with this result, Khalafalla and Daffalla (2008) reported similar observation in *Acacia senegal.* Supplementing the media with BAP evoked shoot proliferation in cowpea with the number of multiple shoots derived increasing with increase in the BAP with or without NAA. The superiority of BAP has also been reported in cowpea (Kononowiez *et al*., 1997, Monti *et al*., 1997, Brar *et al*.,1999) and for other *Leguminous* species (Badji *et al.* 1993., Sahoo and Chand, 1998, Khalafalla and Daffalla, 2008). Induction of multiple shoots from the shoot apices of *Vigna unguiculata* could be related to its activity on apical meristem. Elevation in the cytokinin level has been demonstrated to result in the formation of ectopic meristems in the leaves of *Arabidopsis thaliana*and in the over expression of KNATI and STM-genes which are important in the regulation of meristem function (Rupp *et al*., 1999).

Although supplementing MS with BAP± NAA significantly increased multiple shoot development from shoot apices of seed derived seedlings of V*igna unguiculata,* no significant effect was recorded on the length of the regenerated plantlets. The highest mean shoot length was recorded when MS was supplemented with 1.5mg/L BAP (2.60) and with 0.1mg/L NAA (2.61). However these treatments were not signicantly different from the control treatment (MS without plant growth regulators), but were significantly higher than MS supplemented with 0.1mg/L NAA alone. These results indicated that although NAA has been associated with cell elongation in tissue culture, its effect on plantlets height is minimal.

The two local genotypes used (Kanannado and Danila) tested in this study gave almost a similar response of multiple shoots regeneration via organogenesis on media containing variable concentration (0.5-1.5mg/L) of BAP with or without 0.1mg/L NAA. The genotypes used in this study showed a remarkable response in terms of percentage regeneration which ranged from 56 % - 100% depending on the genotype and the concentration of BAP. This result is however in contrast of earlier report of only 1-11 percentage (Machuka *et al*, 2000).
The effect of the interaction between the hormone supplement and genotype on *in vitro* shoot number and shoot length showed that hormones supplementation within the range of concentration used in this study (0.5 -1.5mg/l BAP $\pm$0.1mg/l NAA) increase multiple shoot production but, do not result in the increase in the *in vitro* shoot length of cowpea in all the genotypes.
Most of the explants formed 1- 3 shoots arising from the basal end of the explants. However, none of these regenerated shoots reached the length of 3cm. Rather basal end of the shoots developed cream coloured callus which eventually covered the whole plantlets suppressing further development. Vengadesan *et al.* (2002) have reported that when cotyledonary nodes explants of *Acacia sinuata* were cultured on MS medium containing a combination of BAP and NAA, the number of multiple shoots increased but in turn produced basal callus.

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