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IN-VITRO REGENERATION OF MULTIPLE SHOOTS FROM APICAL MERISTEMS OF COWPEA (*Vigna unguiculata* L. (WALP) USING DIFFERENT GENOTYPES (LOCAL AND IMPROVED) AND MEDIA COMPOSITIONS

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ABSTRACT

Organogenesis *in-vitro* was evaluated in explants derived from shoot apices of four 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 multiple 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. The highest shoot number was recorded with IT99k-573-2-1 and Danilla and shoot length was highest in Kanannado, IT04k-33-2-1and Danilla indicating that Danilla responded well to *in vitro* regeneration. To optimise the production of multiple shoots the effects of different concentratios (0.5, 1.0 and 1.5mg/L) of 6-benzyaminopurine (BAP) with or without 0.1mg/L α-naphthaleneacetic acid on the number of responding explant, number of multiple shoot produced and length of the shoots were tested. after six weeks of culture, the percentage of responding shoot apices was highest on media supplemented with 1.5mg/L BAP with and without 0.1mg/L NAA.The number of responding apices decreasing with decrease in the concentration of BAP. Maximum number of multiple shoots and the highest mean shoot length were also obtained when MS was supplemented with 1.5mg/L BAP with and without 0.1mg/L NAA.

Keywords: Tissue Culture, Organogenesis, 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 years (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 has considerably adaptation in high temperature and drought compare to other crop species (Hall, *et al* 2002).

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 (Chowrera, *et al* 1995). 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 (Brar, *et al* 1999). India in particular and Asia in general have been proposed to be the third centers of diversity. The cultivated *anguiculata* is the most diverse of the cultivated sub – species *unguiculata*. Varieties may be prostrate, semi erect, erect or climbing and may be determinate in which the apical meristem remains vegetative

Plants have a deep tap root system with a well nodulated lateral root (Singh, 2000).

Leaves are trifoliate although some unifoliate ones have been reported (Christou P., 1997). The leaves subtended by large stipules are supported by petioles which may or may not be pigmented and are about 5 – 15cm long. The flowers are produced in inflorescences that are compound recemes of several modified simple recemes, and majority of flowers are self pollinated (Singh, 2000).

Flowers are cleistogamous and four principal colours are recognized as dark, pale, tinged and white. The purple or violet colouration of cowpea flowers is as a result of high concentration of anthocyanin.

The pods are borne on peduncles that are from less than 5cm to more than 50cm long (Khalallafa, *et al* 2008). The majority of peduncles arise from axillary nodes in which only one of the three buds present normally develops (Singh, 1999). Pods may be coiled, crescent, round or linear and may be partially or wholly purple containing anthocyanin.

MATERIALS AND METHODS

Plant Materials

Four (4) varieties of cowpea comprising two improved (IT 99K-573 – 2- 1 and IT 04K – 332 – 1) and two local (Kanannado and Danila) obtained from International Institute of Tropical Agriculture (IITA) Kano, were used as donor materials for this study.

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, 1976) 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 121^{o}C. All cultures were incubated at 27 $\pm$ 2^{o}C.

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 $\pm$ 2^{o}C, 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 (Kalallafa, *et al* 2008).

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 $\pm$ 2^{o}C under 16hrs photoperiod. Number of explants growing multiple shoot, number of shoots per explant and mean length of shoots were recorded for each treatment (Anand, *et al* 2001). ANOVA was used in mean separation. Mean that has less than 0.05 indicate significance difference and vis-a-visa.

RESULTS

Shoot development was recorded in 50% of the induction media after two weeks of incubation under sixteen hours photoperiod. Single shoot development was observed in 70% of the cultures containing control media (0mg/l BAP) after three (3) weeks of incubation. Development of multiple shoots ranging from 2 to 4 shoots in few cultures (and about 5 shoots in four (4) cultures) containing (0.5-1.5mlg) with or without 1.0mg/l NAA was observed after three weeks of culture.

Statistical analysis showed that there were significant ($P < 0.0001$) differences among the hormone supplements, and genotypes in terms of the number of shoots produced. However, no significant difference was observed among the experiment replications and interactions between the hormone supplements and genotypes (Appendix 1).

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%. Danila was closely followed by IT04K-332-1 and IT99K-573-2-1 with *in vitro* response of 87% and 85% respectively. The least among the genotypes was Kanannado with 75% response.

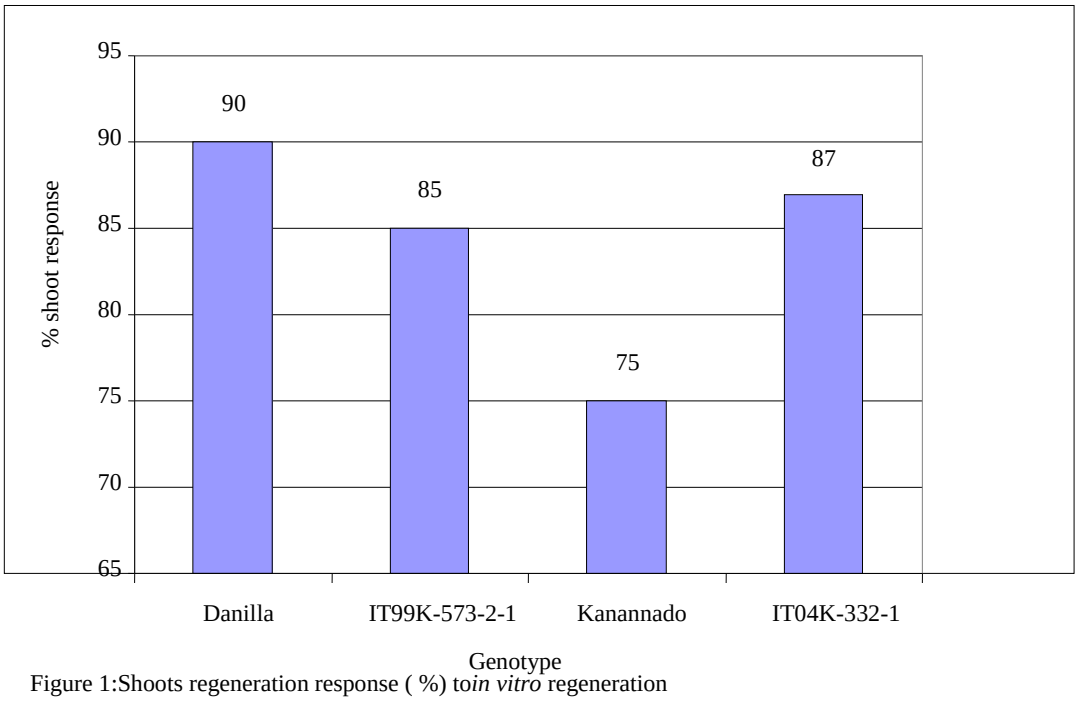

Figure 1:Shoots regeneration response (%) to*in vitro* regeneration

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 .

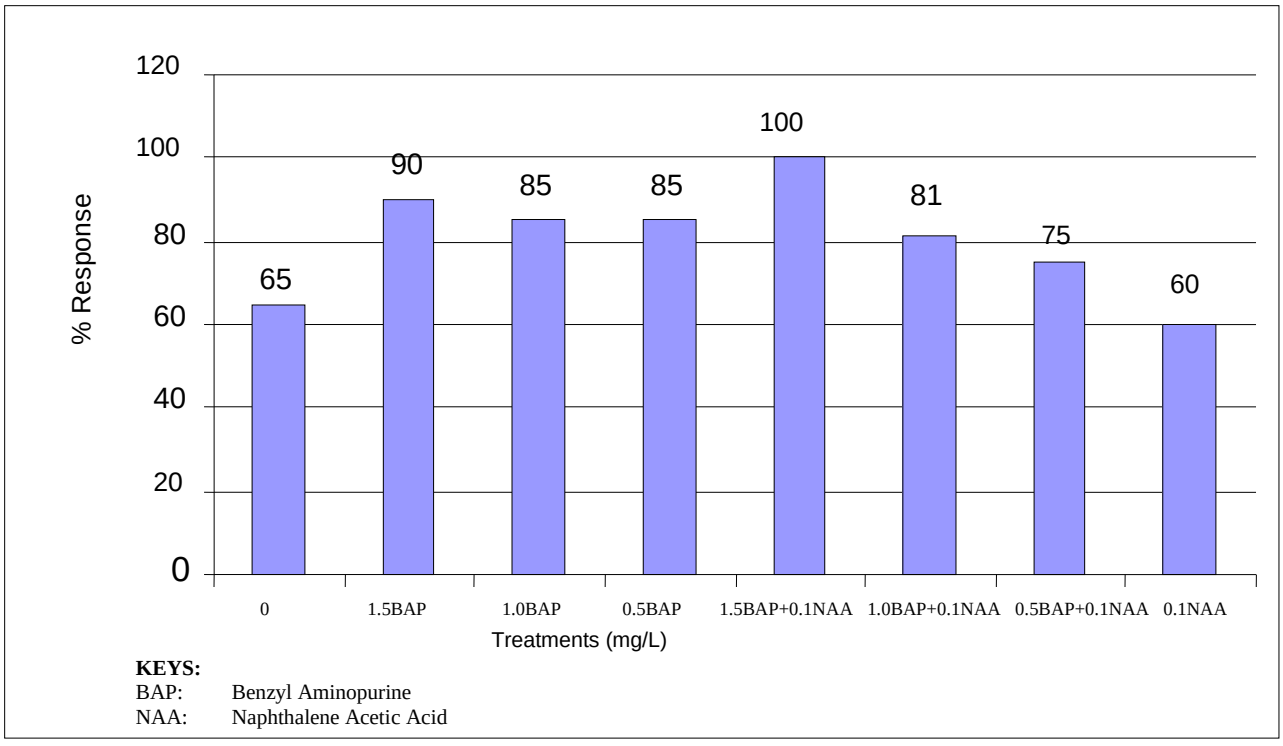

Figure 2: Percentage regeneration of the Cowpea Genotypes to *in vitro* regeneration under different treatments.

The genotype X treatment interaction on the number of explants responding to the induction treatment was also studied (Appendix II) . The highest percentage of responding explants was recorded on media supplemented with 1.5mg/l BAP + 0.1mg/l NAA (100%) in Dan'ila, Kanannado and IT99K.-573-2-1. In all the three (3) genotypes, the number of responding explants decreased with decrease in the concentration of BAP with or without 0.1mg/L NAA. However, IT04K-333-2 demonstrated a different pattern in its response to *in vitro* regeneration in the presence of BAP with

or without NAA. This genotype produced the highest response in media supplemented with 1.5mg/L BAP alone.

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 ± 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.

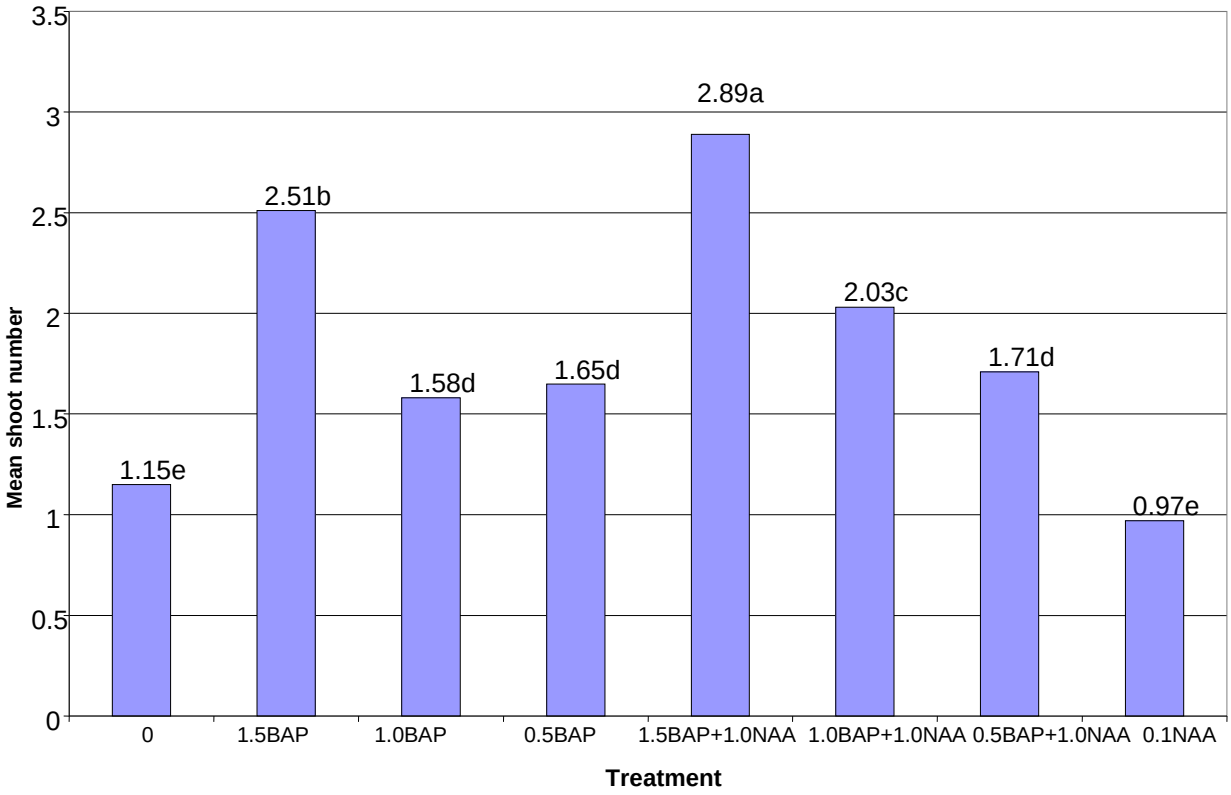

Figure 3: Effect of Hormone Supplementation (BAP ± NAA) on *in vitro* Shoot Number in cowpea explants on MS media

The effect of genotype on multiple shoot production is also illustrated in Figure 4. IT99K produced the highest mean shoot number (1.96a) and was not statically different from Dan'ila (1.89ab). However, these two genotypes were significantly higher than IT04K – 332-1 (1.69c) and Kanannado (1.74bc).

Appendix III shows the interaction between the hormone supplement and cowpea genotypes on the number of shoots produced. While highest number of multiple shoots in IT99k-573-2-1 and IT04k-332-1were produced when MS was supplemented with 1.5mg/L BAP alone, the local cultivars (Danilla and Kanannado) produced the highest number of multiple shoots on MS supplemented with 1.5mg/L BAP + 0.1mg/L NAA. Although multiple shoot production increased with increase in the BAP concentration, the genotypes were observed to responded well when MS was supplemented with BAP + NAA.

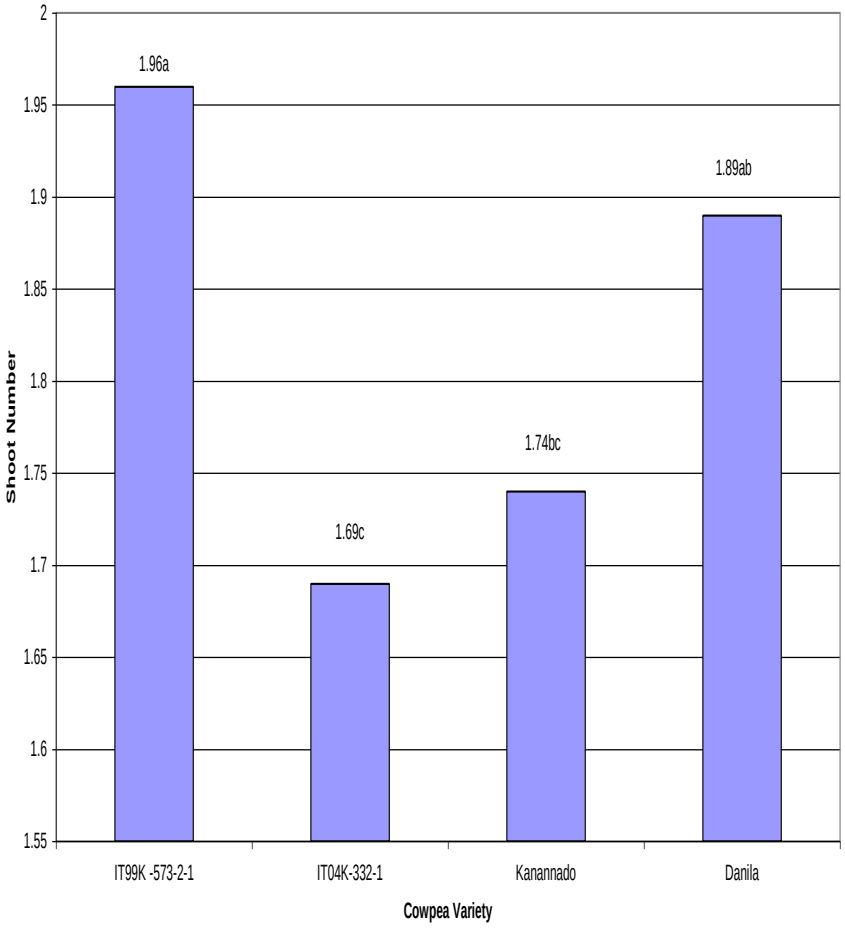

Figure 4: Response of Cowpea Genotypes to *in vitro* shoot production on MS media ranging from (0.5-1.5)BAP ± (0.1) NAA

Appendix IV is the ANOVA table for mean shoot length. The table shows that there was a highly significant difference between the hormone supplement (P<0.0001), the genotypes (0.0061) and the interaction between the hormone supplement and the genotypes.

The effect of hormonal supplement on mean shoot length is shown in Figure 5. The highest shoot length was obtained when media were supplemented with 1.5mg/l BAP alone (2.60a) and with 0.1mg/l NAA (2.61a) which were not significantly different from 1.0mg/l BAP alone (2.52ab) and with 0.1mg/l NAA (2.49ab), 0.5mg/l BAP alone (2.52ab) as well as the control (2.37ab). Supplementing media with 0.1mg/l NAA produced mean shoot length (1.94c) that was significantly lower than the control treatment (2.37ab).

The response of cowpea genotypes in term of shoot length is also evaluated Fig.5. 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).

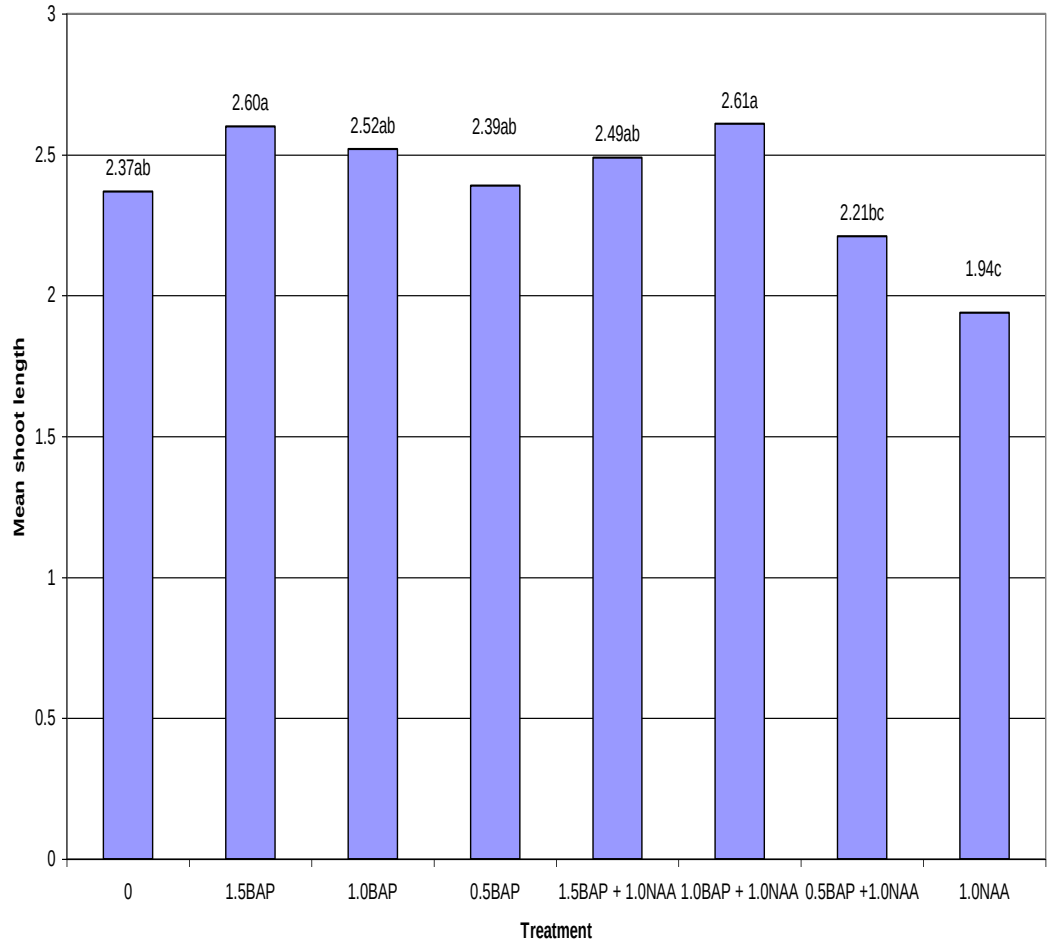

Figure 5: Effect of Different Concentration of BAP ± NAA on *In vitro* shoot length in cowpea explants**.**

The effect of the interaction between the hormone supplement and genotype on *in vitro* shoot length is shown in Appendix V. Hormone supplementation with the range of concentration used in this study (0.5 -1.5mg/l BAP ± 1.0mg/l NAA) do not result in the increase in the *in vitro* shoot length of cowpea in all the genotypes. However, 0.1mg/L NAA resulted in the decrease in the *in vitro* shoot length of cowpea producing shoot length lower than control in all the genotypes tested in this study.

The response of cowpea genotype to mean shoot length was evaluated in figure 6. The genotype that recorded the highest mean shoot length is Kanannado (2.49a) fallowed by Dan'ila (2.47a). IT 04k- 332-1 has mean shoot length of (2.43a) which was higher than that of IT 99k – 573 -2 -1, (2.17b). There was no significant difference between Kanannado, Danila and IT 04k in relation to mean shoot length. There was a significant difference between IT 99k and other genotypes.

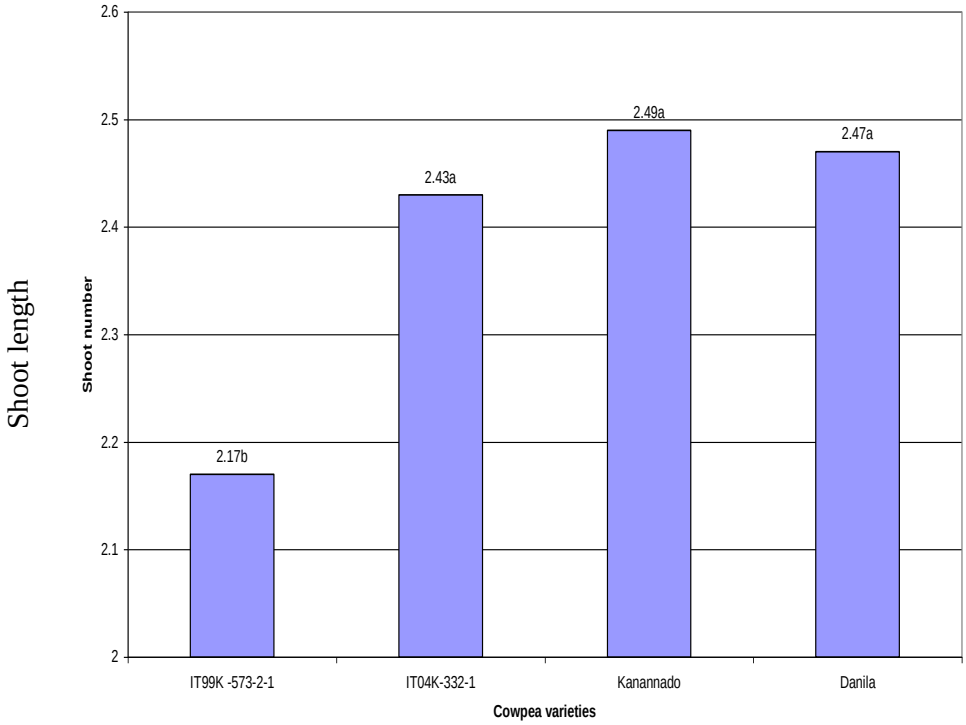

Figure 6: Response of Cowpea genotypes to mean shoot length

The effect of interaction between hormone supplement and genotype on *in vitro* shoot length is shown in Appendix V. These supplementation (0.5 – 1.5 mg/l BAP ± 0.1mg/l NAA) do not result in the increase of in vitro shoot length of cowpea in all the genotypes. However , 0.1mg/l NAA resulted in the decrease of the *in vitro* shoot length of cowpea.

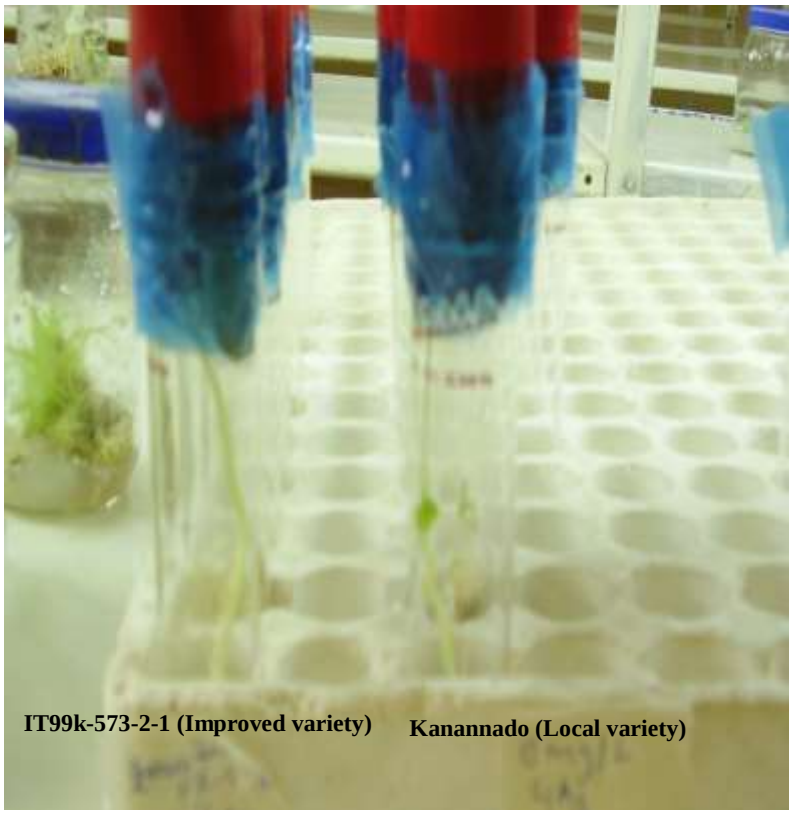

Plate1: Two (2) weeks old seedlings from excised cowpea embryos, cultured on MS basal medium.

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.

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* (Estruch *et al*.,1991) 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 four genotypes (IT99K-573-2-1, ITo4K-332-1, 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.

CONCLUSION

In conclusion, induction of multiple shoots on MS supplemented with 1.5mg/L BAP + 0.1mg/L NAA, was achieved in both improved and local cowpea varieties used in this study. 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. The objective the study is achieved as multiple shoot are produced using MS supplemented with 1.5mg/L BAP+0.1mg/L NAA. It could also serve as a spring board for further research on the *in vitro* transformation of important traits in cowpea.

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APPENDICES

APPENDIX I: ANOVA Table for Shoot Number

Sources	DF	Type III SS	Mean Square	F Value	PR >F
Replication	17	11.030	0.65	1.21	0.2493
Supplement	7	209.082	29.87	55.81	<.000
Genotype	3	6.71	2.24	4.18	0.0061
Supleme or Genotype	21	18.27	0.887	1.63	0.0392

SAS System 12:31 12

APPENDIX 1I: Response of Cowpea Explants to Mutiple Shoots Production

Genotypes	Treatments							
	1.00 (%)	1.5 BAP(%)	1.0 BAP(%)	0.5 BAP(%)	1.5 BAP + 1.0 NAA (%)	1.0 BAP + 1.0NAA (%)	0.5 BAP + 1.0 NAA (%)	0.1mg/l NAA (%)
Danlila	5.6	88.9	61.1	72.2	100	77.8	55.6	22.2
Kannanado	22	66.7	66.7	61.1	100	66.7	44.4	11.1
IT99K – 573-2 – 1	33	83.3	44.4	38.9	100	77.8	61.1	22.2
IT 04K – 332 – 1	5.6	94.4	55.6	55.56	88.9	72.2	44.4	5.6

APPENDIX III: Response of Cowpea Genotype to In – Vitro Shoot Number Under Different Hormone Regimes

PGR Conc. (Mg/l/L)	Mean Shoot number			
	IT99K – 5732 – 1	IT04 K – 332 – 1	Kannanado	Danila
0.00	1.39±0.61	1.06±0.24	1.17±0.51	1.00±0.34
1.5mg/l Bap	3.06±1.21	2.56±0.92	2.06±0.99	2.39±0.85
1.0mg/l BAP	1.50±0.61	1.56±0.51	1.67±0.49	1.61±0.50
0.5mg/l BAP	1.44±0.62	1.61±0.61	1.67±0.77	1.89±0.68
1.5mg/l BAP + 1.0mg/l NAA	2.94±0.73	2.44±0.70	2.89±0.90	3.28±1.07
1.0mg/l BAP + 1.0mg/l NAA	2.39±1.04	1.94 ± 0.80	1.83 ± 0.71	1.94±0.64
0.5mg/l BAP + 1.0mg/l NAA	1.83+0.79	1.56±0.70	1.61±0.71	1.83±0.92
0.1mg/l NAA	1.11±0.58	0.78±0.55	1.00±0.49	1.06±0.64

APPENDIX IV: ANOVA TABLE FOR SHOOT LENGTH

Sources	DF	Type III SS	Mean Square	F Value	PR >F
Replication	17	25.29	1.48	1.94	0.0133
Supplement	7	25.57	3.65	4.78	<.0001
Genotype	3	9.27	3.09	4.04	0.0074
Suppl. Genotype	21	11.46	0.55	0.71	0.8215

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APPENDIX V: Response of Cowpea Genotype to *In – vitro* Shoot Length Under Different Hormones Regimes.

PGR Conc. (Mg/l/L)	Mean Shoot number			
	IT99K – 57 – 32 – 1	IT04 K – 332 – 1	Kannanado	Danila
0.00	2.22±0.81	2.56±1.06	2.56±0.84	2.17±0.86
1.5mg/l Bap	2.16±0.63	2.55±0.54	2.81±1.06	2.77±1.05
1.0mg/l BAP	2.30±0.79	2.68±0.75	2.69±0.94	2.41±0.86
0.5mg/l BAP	2.07±0.64	2.62±1.10	2.24±0.83	2.62±0.86
1.5mg/l BAP + 1.0mg/l NAA	2.49±1.05	2.31±0.95	2.55±1.20	2.61±1.13
1.0mg/l BAP + 1.0mg/l NAA	2.25±0.81	2.82±1.06	2.91±1.07	2.75±1.00
0.5mg/l BAP + 1.0mg/l NAA	1.96 ± 0.66	2.18±0.86	2.23±0.89	2.46±1.11
0.1mg/l NAA	1.93±0.60	1.91±0.63	1.95±0.53	1.95±0.61