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Media Optimization and Effects of Benzylaminopurine and Gibberellic acid on Shoot Regeneration of *Capsicum annuum* L. (Cayenne)

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ABSTRACT

The development of an efficient *in vitro* propagation protocol for *Capsicum annuum* becomes necessary in order to boost its seedlings availability. The effects of Murashige & Skoog (MS) medium strengths (0, 25, 50, 75 and 100 %) and different combinations of Benzylaminopurine (BAP)/Gibberellic acid (GA_3) in order of 0/0, 1.0/2.0, 2.0/1.5, 3.0/1.0 and 4.0/0.5 mg/L were assessed on *in vitro* seed germination and shoot regeneration of the species respectively. In all cases, media without hormones (0/0) serve as the control for this study. The set of treatments were laid out in CRD with each having 10 replicates. The results showed that all the media strengths and control supported the germination of *C. annuum* seeds under first Weeks After Inoculation (WAI). However, the assessed morphological parameters of the plantlets showed that optimum growth was obtained in 25% MS having the highest shoot lengths (4.97 cm) and number of leaves (2.4) at 3 WAI. The control medium had the highest mean number of roots (1.8) and root length (3.34 cm) at same period. The results of BAP/GA_3 combinations on the regenerated plantlets revealed that control (no supplement) supported highest growth indices and elicited best plant responses compared with other treatments. Plantlets from control had significant higher ($p = 0.05$) mean number of leaves (5.83), shoot lengths (3.17 cm), root lengths (4.37 cm), number of roots (6.83) at 8 WAI. The regenerated plantlets were acclimatized in the green house with 80% plant survival rate. The developed protocol is sustainable and shows a promising result that could support mass propagation of seedlings of *Capsicum annuum*.

Keywords: Inoculation, *In vitro* propagation, Seed germination, Shoot regeneration

Introduction

Capsicum annuum commonly called pepper belongs to the family Solanaceae. It is a tropical species which originated from Mexico in America and is the most widely cultivated among the five domesticated species of capsicum. It is an important agricultural crop with a lots of economic, nutritional and medicinal values (Alawode and Abegunde, 2016). Generally, *Capsicum spp* serves as a source of natural colours and antioxidant compounds in meals and snacks. Among the essential compounds found in the species is Capsaicin, which exhibits anti-oxidant, anti-mutagenic, anti-carcinogenetic and immune system modulating activities (Sreshtaa *et al*., 2020).

Pepper is consumed in large quantity in Nigeria especially in the south western part. Hot pepper is considered as standard African vegetable which makes it to be widely cultivated in Africa. Whereas, sweet pepper is seen as an exotic species introduced by the Europeans (Grubben and Mohamed, 2004). The cultivation of pepper in the region is limited by a range of factors which include poor agricultural production facilities, low soil fertility, weeds,

unfavourable weather conditions, pest and diseases, poor yields and high cost of seeds with low viability (Ogunbo *et al*., 2015; Bortolotti *et al*., 2002).Consequently, the need to encourage more participation in the cultivation of pepper in large quantity in the region requires availability of improved cultivars through plant biotechnology techniques (Ochoa-Alejo and Malagon, 2001).

Plant Tissue Culture otherwise known as *in vitro* or micro propagation is a technique with potentials for cell behaviour management, plant modification and improvement, production of pathogen-free plants, germ plasm storage and mass propagation of plants. Other advantages of this technique include regeneration of whole plant from different plant parts such as seed, embryo, stem and leave while making improved plant seedlings available for farmers irrespective of weather conditions. Therefore, application of this technique in pepper propagation can increase the pest and disease resistance characteristics of the species while improving yield (Grubben and Tahir, 2010). Nonetheless, prior to improvement, procedures for *in vitro* regeneration and mass propagation of the improved cultivars has to be developed. Capsicum members have been reported to be recalcitrant to differentiation and plant regeneration under *in vitro* conditions thus, limiting its mass propagation (Ochoa-Alejo and Malagon, 2001). In the light of the above, the present study sought to develop protocol for culture establishment, shoot regeneration, root induction and acclimatisation of *capsicum annum* plantlets with a view for mass propagation.

Materials and Methods

This study was carried out in the Department of Biotechnology, Forestry Research Institute of Nigeria, Jericho, Ibadan, Oyo State, located on longitude 07°23′18″ to 07°23′43″N and Latitude 03°51′20″ to 03°23′43″E.

Culture initiation

Different Murashige & Skoog (MS) media strengths: control (distilled water), 25, 50, 75 and 100 %were used for germination of *C. annuum* seeds.MS basal media (100 %) was prepared using 34.43 g/L of MS power (M5501, SXS5501015A). The pH of the media was adjusted to 5.8and added 8.5 g/L of agar (J.T Baker/4068), homogenized in hot air microwave oven and dispensed at 20ml/test-tube. The tubes were labelled according to treatments and was sterilized using autoclave at 121°C and 15psi for 15 minutes. Other materials including forceps, scalpels, petri-dishes and filter papers were also sterilized at the same condition. The sterilized media was transferred to Laminar air flow hood for inoculation.

Seed of *C. annuum* was procured from an Agro allied store in Ibadan. Firstly, the seeds were treated with 70 % ethanol for 5 minutes followed by a mixture of fungicide/antibiotics for 30 minutes and then10% sodium hypochlorite for 15 minutes. Each step was followed by rinsing thrice with sterilized distilled water before the next. The seeds were blotted on sterile filter paper laid petril-dish and then inoculated at three to four seeds per tube.

Shoot regeneration

Murashige & Skoog basal media was prepared and supplemented with Benzylaminopurine (BAP) and Gibbrellic acid (GA_3) in order of 0/0, 1.0/2.0, 2.0/1.5, 3.0/1.0 and 4.0/0.5 mg/L respectively. The media was sterilized using the same condition as stated before. The explants used for this stage were obtained from the seed plantlets of the previous experiment (Plate 1a). About 1.5 cm of the shoots tips of the plantlets were excised and inoculated into the prepared media. Each stage of the

study consist of five set of treatments and were laid out in completely randomized design with 10 replicates.

Data collected include number of leaves, shoot length, number of root and root length at 3 WAI for cultured seeds and at intervals of two weeks for regenerated shoots. The data were subjected to analysis of variance and significantly different means were separated using fisher's protected least significant difference (L.S.D) @ p = 0.05.

Acclimatization of regenerated C. annuum plantlets

Shredded dried coconut husk mixed with topsoil (ratio 3:1) was moisten with distilled water, bottled and sterilized using autoclave at 121°C and 15psi for 15 minutes. The tubes containing the regenerated plantlets of *C. annuum* were de-capped, sprayed with fungicides and placed under laboratory conditions for three days (Plate 1b).Then, the plantlets were carefully removed, have their roots rinsed and then transplanted into acclimatization trays filled with the prepared acclimatization media (plate 1c). The trays were first kept under mist propagator for 5 days and were later exposed to acclimatization chamber condition.

Results and Discussions

Culture establishment

This study involved media optimization for *in vitro* seed germination of *Capsicum annuum.* The results are presented in Figures 1 and 2. Observations made on the germination of seeds showed that there was 100% germination across the treatments and control hence, plantlet growth attributes were used to identify the best medium. The results of analysis of variance conducted on the growth attributes showed that there was significant difference (p = 0.05) between the means for shoots and roots length (Figure 1). In all media treatments, similar number of leaves were recorded while the number of roots showed variation to the control 3 Weeks After Inoculation (WAI) (Figure 2).

The longest shoot length (4.97 cm) produced by 25 % MS was comparable to values for other media strengths but were all significantly higher than control which gave the least (3.34 cm) at 3 WAI (Figure 1). Conversely, the longest average root length (3.34 cm) was obtained from control which was closely related to 3.09 cm, 2.72 cm and 2.69 cm gotten from 25 %, 75 % and 100 % MS respectively at 3 WAI (Figure 1). The shortest root length (1.65 cm) was obtained from 50 % MS media at same period. Similarly, highest average number of roots (1.8) was obtained from control which was significantly higher than values from all the MS media strengths from among which there was no significant different (Figure 2). Moreover, the results for the number of leaves of the seed plantlets ranged from the highest (2.2 leaves) in 25 % MS to the lowest (2 leaves) in 75 % MS at 3 WAI (Figure 2).

Journal of Forestry Research and Management. Vol. 19(2).62-71; 2022, ISSN 0189-8418
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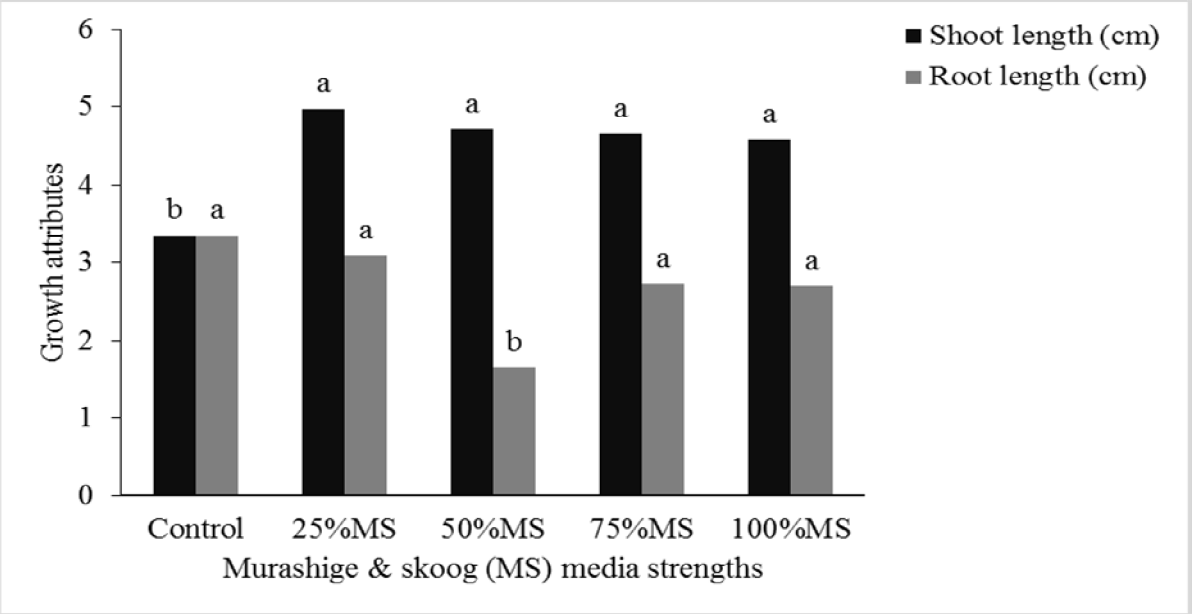

Figure 1: Effects of media strengths on shoot length and root length of *Capsicum annuum* plantlets generated from seeds at 3 weeks after inoculation.

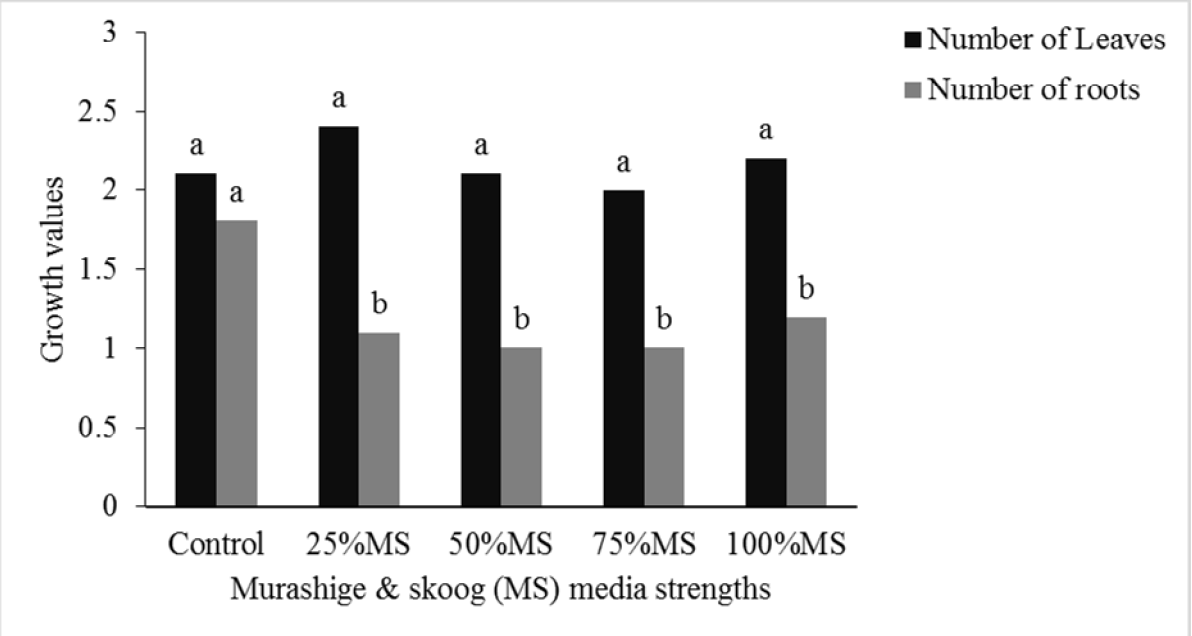

Figure 2: Effect of media strength on number of leaves and roots of *Capsicum annuum* plantlets generated from seeds at 3 weeks after inoculation.

The observed germination of the pepper seeds from control and the different MS media used under 3 WAI indicates that the seeds does not require the MS media nutrients for germination (Figures 1 and 2). These results emphasised the need for only water and air to initiate germination processes in the seeds. This was evident in the higher morphological growth by number of roots (Figures 2) and root length (Figures 1) from the plantlets in control medium compared with MS media. However, the higher shoot growth obtained across the MS media compared with control at 3 WAI

could be attributed to root facilitated uptake of available nutrients in the MS media which was absent in control. Nonetheless, the higher shoot length (Figures 1) and number of leaves (Figures 2) obtained from 25 % MS compared with other MS strength indicated that 25 % MS medium was the optimum for pepper seeds germination. These results could be related to that of Ebida and Hu (1993) which stated that seeds of *C. annuum* were germinated in 50 % MS medium without growth regulators. Moreover, the result was an improvement on that of Swamy *et al*. (2014) who achieved pepper seed germination on 100 % MS medium supplemented with BAP and kinetin in their preliminary study.

Shoot regeneration

The regeneration and growth of *C. annuum* as affected by BAP and GA_3 combinations were assessed. Analysis of variance conducted on number of leaves of the plantlets revealed that values for the growth attribute were comparable at 2 WAI whereas, there was significant variation (p = 0.05) between the treatments at 4, 6 and 8 WAI (Figure 3). Moreover, the results at those periods of growths followed similar trends. The control medium (MS basal with no growth regulators) elicited highest number of leaves having 3.8, 5 and 5.8 average leaves which were closely related to 3.1, 4.5 and 4.6average leaves obtained from MS medium supplemented with 1.0/2.0 BAP/GA_3at 4, 6 and 8 WAI respectively (Figure 3). At the same time, the number of leaves produced by other treatments were significantly lower than control while MS medium added with 3.0/1.0 BAP/GA_3gavethe least (2.3 and 2.3) at 6 and 8 WAI respectively.

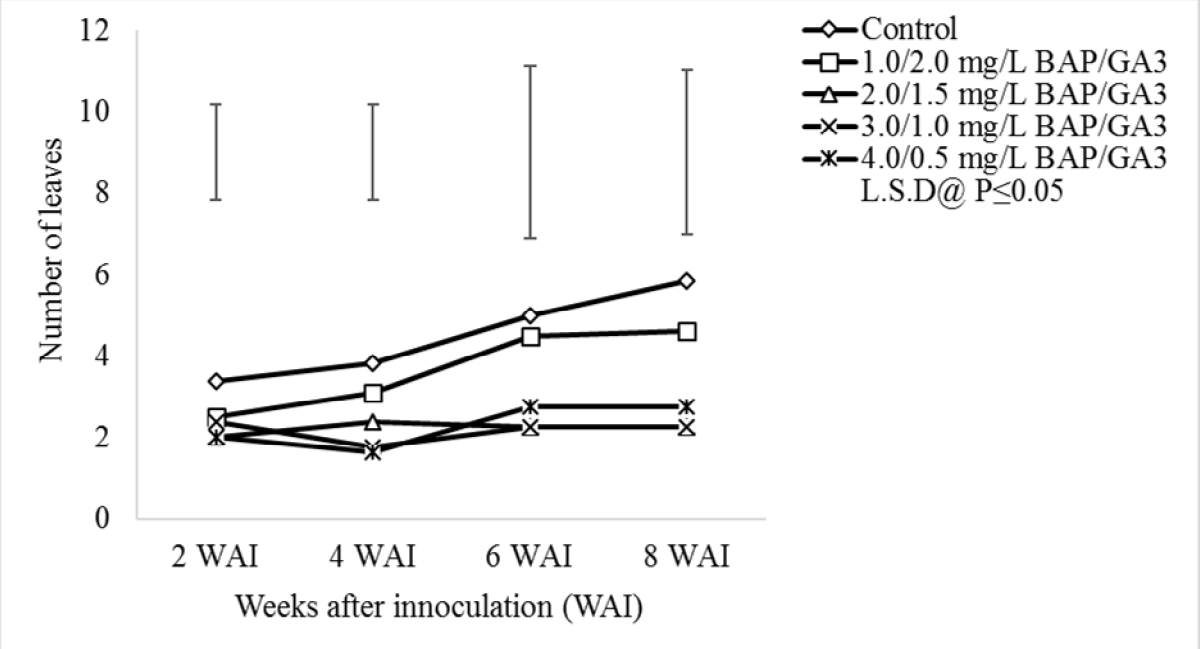

Figure 3: Effects of BAP/GA_3 combination on the number of leaves of regenerated plantlets of *Capsicum annuum* at successive weeks after inoculation.

The results of the shoot length of the regenerated *C. annuum* indicated that there was significant difference (P=0.05) in the effects of the growth regulators on the attribute at successive growth weeks. The shoot length of plantlets from control (MS

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medium without growth regulator) was the longest and comparable to those of MS media added with 1.0/2.0 mg/L BAP/$GA_3$and 3.0/1.0 mg/L BAP/GA_3while it was significantly higher than MS media supplemented with 4.0/0.5 mg/L BAP/$GA_3$and 2.0/1.5 mg/L BAP/GA_3at 2 and 4 WAI (Table 1). The results at 6 and 8 WAI followed similar trends, as average shoot length from control medium was comparable to MS media enhanced with 1.0/2.0 mg/L BAP/GA_3while it was higher than others treatments at both periods of investigation. The least shoot length was obtained from MS medium added with 2.0/1.5 mg/L BAP/GA_3across the successive growth weeks.

Table1.Effectsof BAP/GA_3 combination on the shoot length of regenerated plantlets of *Capsicum annuum* at successive growth weeks

Treatment (BAP/GA_3 mg/L)	Shoot length (cm)			
	2 WAI	4 WAI	6 WAI	8 WAI
Control	2.03a	2.28a	3.00a	3.17a
1.0/2.0	1.93ab	2.09ab	2.60ab	2.65ab
2.0/1.5	1.61c	1.68c	1.36c	1.45d
3.0/1.0	1.86ab	1.96abc	2.14b	2.15bc
4.0/0.5	1.75bc	1.88bc	1.88bc	1.91cd
L.S.D@ P=0.05	0.25	0.33	0.75	0.66

Means in the same column followed by the same alphabet(s) are not significantly different @ p = 0.05. WAI: weeks after inoculation

The results of number of roots of the regenerated *C. annuum* plantlets showed that the growth regulators did not exert significant positive influence on the attribute at successive growth weeks. The control medium had significantly higher number of roots throughout the periods of investigation as presented in Table 2.Other treatments failed to induce roots at 2 and 4 WAI while the few roots produced at 6 and 8 WAI were comparable (Table 2 and Plate 2). Similarly, the results of root length showed that plantlets from control medium had significantly longer roots compared with other treatments across successive growth weeks (Table 3).

The results of acclimatization efforts showed that pepper plantlets transplanted andexposed to acclimatization chamber condition had 80 % survival (Plate 1d).

Table 2: Effects of BAP/GA_3 combination on number of roots of regenerated plantlets of *Capsicum annuum* at successive growth weeks.

Treatment (BAP/GA_3 mg/L)	Number of roots			
	2 WAI	4 WAI	6 WAI	8 WAI
Control	3.4a	4.4a	6.2a	6.8a
1.0/2.0	0.0b	0.0b	1.4b	2.0b
2.0/1.5	0.0b	0.0b	0.0b	2.0b
3.0/1.0	0.0b	0.0b	0.0b	0.0b
4.0/0.5	0.0b	0.0b	0.5b	0.5b
L.S.D @ P=0.05	0.59	0.54	1.55	2.46

Means in the same column followed by the same alphabet(s) are not significantly different @ p = 0.05. WAI: weeks after inoculation

Table 3: Effects of BAP/GA_3 combination on root length of regenerated plantlets of *Capsicum annuum* at successive growth weeks.

Treatment (BAP/GA_3 mg/L)	Root length (cm)			
	2 WAI	4 WAI	6 WAI	8 WAI
Control	1.64a	3.62a	4.17a	4.37a
1.0/2.0	0.00b	0.00b	0.88b	1.60b
2.0/1.5	0.00b	0.00b	0.00c	0.95bc
3.0/1.0	0.00b	0.00b	0.00c	0.00c
4.0/0.5	0.00b	0.00b	0.19bc	0.21bc
L.S.D@ P=0.05	0.20	0.48	0.84	2.46

Means in the same column followed by the same alphabet(s) are not significantly different @ p = 0.05. WAI: weeks after inoculation

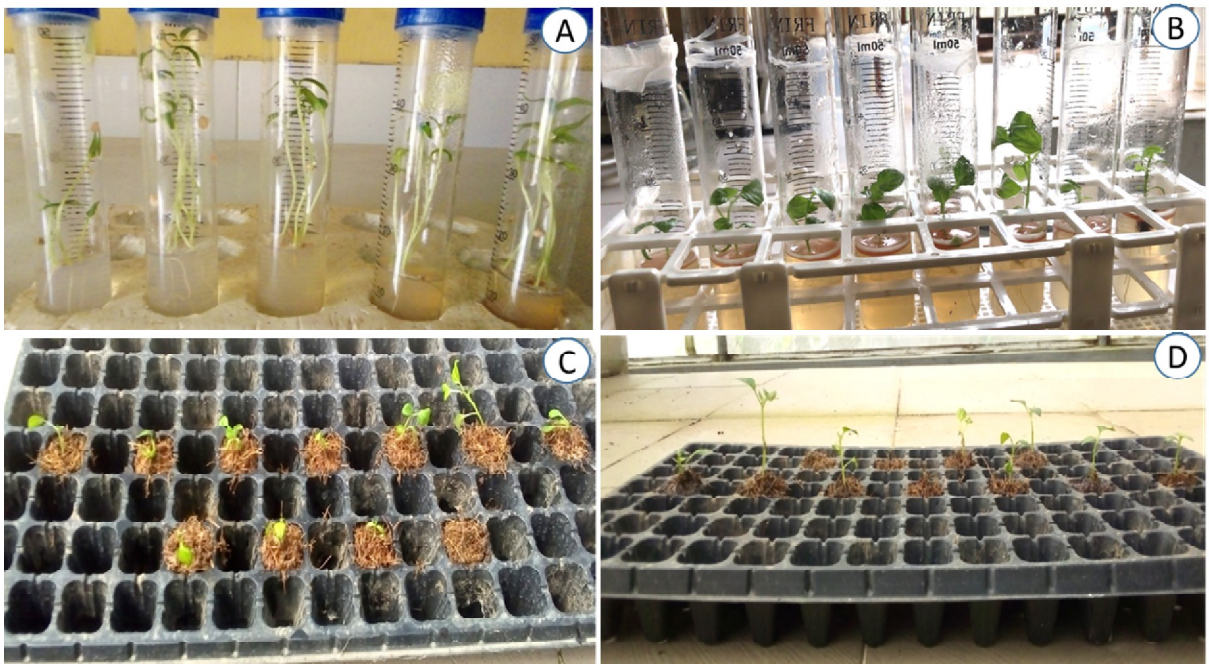

Plate 1a: Plantlets of *Capsicum annuum* generated from seeds; **b**: Regenerated plantlets of *C. annuum* exposed to room conditions; **c**: Transplanted *C. annuum* plantlets at first day of transplanting; **d**: Acclimatized *C. annuum* seedlings at 3 weeks after transplanting.

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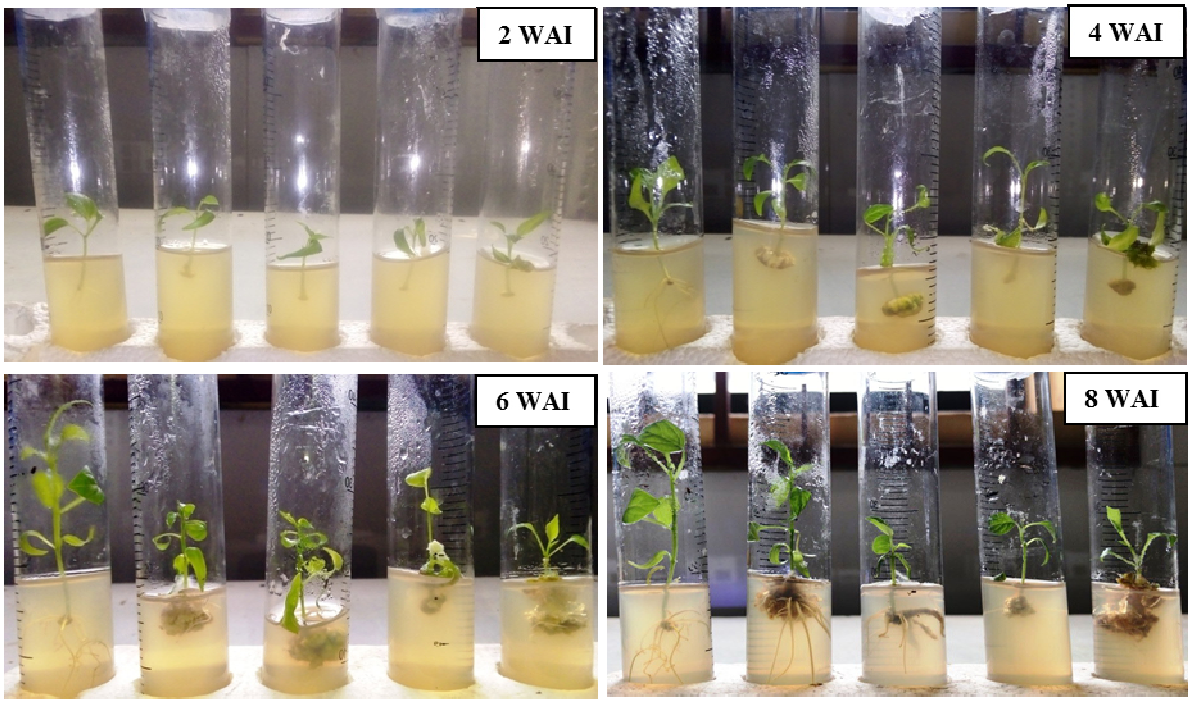

Plate 2. Effects of BAP/GA_3 combinations on regeneration of *Capsicum annuum* plantlets at successive growth weeks.

Tubes from L-R: A: Control, B: 1.0/2.0 mg/L BAP/GA_3, C:2.0/1.5 mg/L BAP/GA_3, D: 3.0/1.0 mg/L BAP/GA_3, E:4.0/0.5 mg/L BAP/GA_3.

Plant growth regulators are excellent inducers of plant regeneration and proliferation (Afolabi *et al*., 2020 a & b). The observed higher morphological growth in *C. annuum* plantlets from the control medium (MS basal without growth regulators) in terms of number of leaves (Figure 3), shoot length (Table 1), number of roots (Table 2) and root length (Table 3) across the successive growth weeks indicated that the species needed no growth regulators for its shoot regeneration. This means that the endogenous cytokinins present in the pepper plantlets was enough to elicit growth without external supplements. This was evident in the better results obtained in terms of number of leaves and shoot length from the lowest combinations of the growth regulators (1.0/2.0 mg/L BAP/GA3) compared with other concentrations which gave poor performance.

Furthermore, this indicated that addition of BAP and GA_3 above 1.0 and 2.0 mg/L exerted limitation on shoot growth while hindering good root formation in the regenerated pepper plantlets (Plate 2). The results of this present study differ from some initial reports on the *in vitro* propagation of pepper where different pepper varieties were best regenerated in the presence of growth regulators (Otroshy *et al*., 2011; Hu *et al*., 2012; Husain *et al*., 1999).This difference might have resulted from the use of different types of varieties, explants, growth regulators, media and their concentrations (Renfiyeni *et al*., 2017). In their reports, Otroshy *et al*. (2011) stated that the use of shoot tips did not produce satisfactory results as normal shoot failed to develop but formed calli after 12 days of culture. This could have resulted from the use of higher concentration of cytokinins as compared to what gave better results in the

present study. Similarly, Ebida and Hu (1993) reported that regenerated shoot-buds of *C. annuum* (Early California Wonder) only produced roots when cultured in full strength MS medium with 0.5 mg/L IAA or 0.4 mg/L NAA which were not considered in the our study. Nonetheless, results obtained in this study could be related to that of Valera-Montero and Ochoa-Alejo (1992), who obtained shoot elongation of *C. annuum* (Salvatierra) hypocotyls bearing buds on full MS medium, free of growth regulators. The results also corresponded to that of Ezura *et al*., (1993), who obtained shoot regeneration and root induction of thirteen cultivars of bell shaped *C. annuum* on MS basal medium without exogenous growth regulators.

Conclusion

The present study was conducted to develop protocol for culture establishment, shoot regeneration, root induction and acclimatisation of *Capsicum annum*. Results showed that the species seed germination requires no or little MS media nutrients (25 % MS basal) for germination while its shoot regeneration was best achieved in full basal media without growth regulators. Moreover, the use of coconut husk and topsoil mixture supported the pepper transplants with 80 % survival under screen house condition. This developed protocol was adjudged to be efficient and reproducible while it offers opportunity for regeneration and mass propagation of pepper. Therefore, the protocol is recommended for *in vitro* propagation of *C. annuum*

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