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Combination of Hairy Roots Explants and 6-Benzylaminopurine (BA) as an Alternative Improvement for *In-Vitro* Plant Regeneration of *Capsicum spp*

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Research Article

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ABSTRACT

Capsicum annuum and *Capsicum frutescens* belong to Solanaceae family are known for tremendous economic importance as crops and medicine. However, *in-vitro* regeneration of Capsicum spp. can be quite challenging. Therefore, in this research study, the uses of hairy roots explants with the 6-Benzylaminopurine (BA) was found highly effective to increase chances during *in-vitro* regeneration of both *Capsicum annuum* and *Capsicum frutescens*. The result had showed that, cotyledon explants that were co-cultured with selective Agrobacterium rhizogenes strain which were ATTC 15834 for *C. frutescens* and ATTC 43056 for *C. annuum* with BA hormones in five different concentrations (0.25, 0.5, 1.0, 1.5, 2.0 mg/L) for two months had promising outcomes for morphogenesis. From the study, the optimum concentrations of BA in *C. frutescens* (1.0 mg/L) and *C. annuum* (1.5 mg/L) had reduced the shoot induction days with lacked of callus formation and necrosis. Even though, the different concentrations of BA able to enhance the direct morphogenesis of both *Capsicum spp*. however there were only slightly morphology differences had been noted in both *Capsicum* species. As a conclusion, the study of using both hairy roots induced cotyledon explants with BA at optimum concentration had shown high potential in regenerating this species by producing leaf, stem and root as well as reducing possibility of recalcitrant.

INTRODUCTION

Most of *Solanaceae* family had economic importance as crops and medicinal plant including *Capsicum.* It can be further classified into the division of Magnoliophyta, class of Magnoliopsida, and order of Solanates [1]. In Asia, *C. annuum* and *C. frutescens* were not only popular as spices and vegetables crops but also as medicine to treat diseases such as analgesia, anticancer, antioxidant, anti-obesity, improved cardiovascular and gastrointestinal activity and reduce osteoporosis [2,3,4]. In field plantation, *Capsicum spp.* have faced many challenges such as viral infection including ring spot, tospo, pepper mottle and also phytopathogenic fungi such as Phytophthora *capsici*, *Fusarium* and *Rhizoctonia* which cause less quality and quantity of *Capsicum* spp. Harvests [5,6]. However, the introduction of *A. rhizogenes* had shown positive respond in improving these plant species by creating better genetic stability and high production of secondary metabolites like capsaicinoids, capsinoids, quercetin, and luteolin [7-9]. Others advantages of hairy roots are also providing a potential development of plant immunity system and also act as phytoremediation [10-12]. The benefits of hairy roots had been proven in many others plants studies such as *Nicotiana tabacum*, *Rubia peregrina L*, *Catharanthus roseus Cicer arietinum*, and *Panax quinquefolius* [13-16]. Therefore, by using *A. rhizogenes* mediated-hairy roots as explant, the possibility chances of recalcitrance during *in-vitro* culture of *Capsicum* spp. hopefully can be reduce. Furthermore, the successful plant regeneration by using this alternative method may have created *Capsicum* spp. plants that possessed similar beneficial properties as shown by previous studies of many other types of plants [17-25]. In this experiment, the application of hairy roots induced explants had become the key factor in selecting the type of plant hormone used. Cytokinin hormone was selected to promote shooting due to present of early rooting cause by hairy roots explants. 6-Benzylaminopurine (BA) was selected due to high success rate noted in previous studies of *Capsicum* spp. [26-33]. However, the compatibility of this hormone towards hairy roots induced explant is yet to be study. Therefore, in this research different concentrations of BA were used to investigate the hormone effect towards direct morphogenesis of both *C. annuum* and *C. frutescens*. Successful regenerated plants with high rate of morphogenesis of *Capsicum* spp. may achieved by optimizing BA concentration as main parameter in this

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study in order to increase regeneration potential while reducing recalcitrance possibility which mainly determined by three main factors which are types of explants, *in-vitro* technique, and present of substance such as plant hormone and media nutrient [34].

MATERIAL AND METHODS

Obtaining Hairy Roots Induced Cotyledon Explants

Seeds of *C. annuum* var cayenne pepper and *C. frutescens* var bird eye's chili were obtained from a local supplier. The seed were surfaced sterilized in 15% sodium hypochlorite, two drops of Tween 80, and 70% ethanol for five minutes and were finally rinsed with sterile distilled water. After two weeks *in-vitro* seed germination, the cotyledon segments were cut and co-cultured with selective *A. rhizogenes strains*, which was ATTC 15834 for *C. frutescens* and ATTC 43056 for *C. annuum* due to similar high rate of hairy roots transformation based on previous study [35]. After a day, the co-cultured explants were decontaminated by using washing media that contained Murashige and Skoog, 1962 (MS) liquid media with carbenicillin disodium (1000 mg/L) for 2 hours before cultured in full strength of MS solid media that contained 500 mg/L with carbenicillin disodium. The explants were maintained by subculture every week in MS solid media containing decreasing concentration of carbenicillin disodium (200,100 and 50 mg/L) for one month.

Preparing Shoot Induction Media

Full strength MS solid media were prepared by adding 4.4 g of MS powder, 1.0 g myo-inositol, and 30 g sucrose, in one liter of sterile distilled water. Then, BA hormones were added respectively in five different concentrations (0.25, 0.5, 1.0, 1.5, 2.0 mg/L) in the media. The media solution pH was adjusted approximately 5.7 to 6.0 by using hydrochloric acid (HCl) or sodium peroxide (NaOH). Then, 4.0 g of gelrite were lastly added in media before been autoclaved in 121°C at 15 psi for 20 min.

Culturing and Maintaining Stage

After one month, the cotyledon induced hairy roots explants were cut and pruning by removing normal roots until the explants only consist of hairy roots. The explants were carefully cultured in jar contain BA treatment media. Each treatment consist 20 replications and the jar were incubated and maintained in 16/8 hours (light/dark) photoperiod for two months under 25°C. The morphology and grow rate of regenerated *Capsicum* spp. were observed.

Data and statistical Analysis

Morphology changes during morphogenesis of both *C. annuum* and *C. frutescens* were observed and measured. The grow rate efficiency was analyzed by two–way analysis of variance (ANOVA) and multiple comparison test were calculated by using Tukey test. All data of mean values with standard deviation were expressed as mean ± SD after two month of growth period.

RESULTS AND DISCUSSION

After two months, the hairy roots induced cotyledon explants had shown positive result in regenerating the whole plants of both *C. annuum* and *C. frutescens* **(Figures 1a and b)**. Different concentrations of BA had shown the ability to enhance the morphogenesis of both *Capsicum* spp. with slightly changes in morphology. Different morphology was shown in *C. annuum* which were found to have reduce in height compared to *C. frutescens* in whole plant structure. In absent or low concentrations BA, the sizes of the leaves were decreased with green coloration. Narrow -pointed leaves shaped **(Figure 2a)** were showed similarly in each BA treatments of *C. annuum* while rounded in *C. frutescens* **(Figure 2b)**. Both *Capsicum* spp. of stem were appeared to have smooth layer without presence of short hair around the stem **(Figure 3a)** with *C. frutescens* stem were thicker compared to *C. annuum* **(Figure 3b)**. There were high numbers of branches present in *C. annuum* compare to lack of branches in *C. frutescens*. There were two types of roots present in both regenerated species which appeared to be a combination of both hairy and normal roots which the hairy roots were more abundant **(Figure 4s a and b)**. Even though, the normal roots were removed before culturing however some root cell due failed to undergoes genetic transformation by *A.rhizogenes* which reported by the previous research studies via *Gus* staining and other molecular evidence supporter [36-42]. The entire roots system of *C. annuum* was appeared to retain their former thicker and shorter root compared to *C. frutescens* which reported on previous study [35]. High concentration of BA (1.5 mg/L and 2.0 mg/L) in both of *Capsicum* spp. had shown capability to induce callus, which mostly located at the roots area (Fig 5). However, the small presence of callus also spotted at the leaves and stem area especially with 2.0 mg/L BA treatment **(Figures 5 a,b,c,d and f)**. Most callus that had been induced at roots area were hard, non-friable with dark brown coloration while the callus induced at stem and leaves were appeared to have better morphology which were soft, friable with white coloration that were more suitable to be used in further tissue culture studies. Low concentration of BA treatment (0.25 and 0.5mg/L) were unsuccessfully induced callus in both species **(Figures 5a and d)**. Most of the callus started to induce after one month culturing process occur in both *Capsicum* spp. which were showed in **Table 1**.

The experiment had showed that, present of BA in MS media did affecting the days of shoot induction in *C. annuum*; however present of BA during shoot induction of *C. frutescens* did not shown any differences. The used of 1.5 mg/L (*C. annuum*) and 1.0 mg/L (*C. frutescens*) BA, had cause the earliest days for shoot induction **(Figure 6)**. However, the increment of BA to 2.0 mg/L had cause delay in shoot induction. Therefore, further increase on BA may reverse the efficiency for shoot induction

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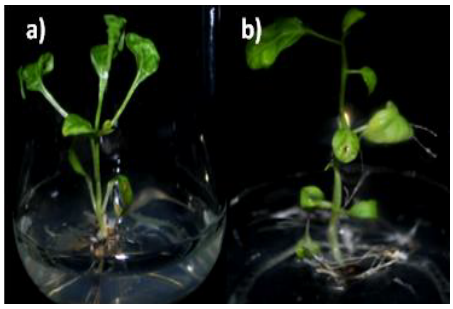

Figure 1. Morphology of whole plants between **a)** *C. annuum* and **b)** *C. frutescens* after 2 months in BA.

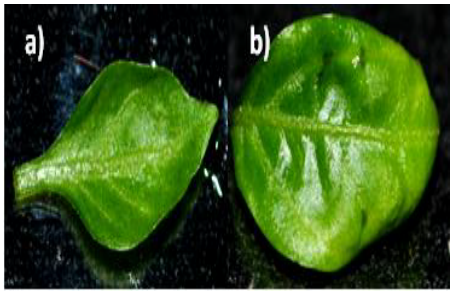

Figure 2. Leaf morphology of **a)** C. annuum and **b**) C. frutescens after 2 months in BA.

Figure 3. Stem morphology of **a)** C. annuum and **b)** C. frutescens after 2 months in BA.

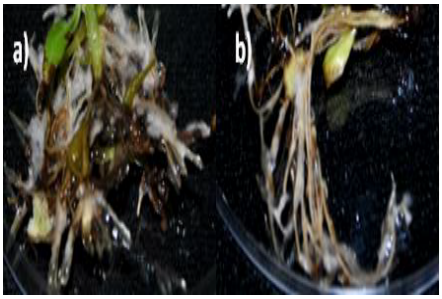

Figure 4. Roots morphology of **a)** C. annuum and **b)** C. frutescens after 2 months in BA.

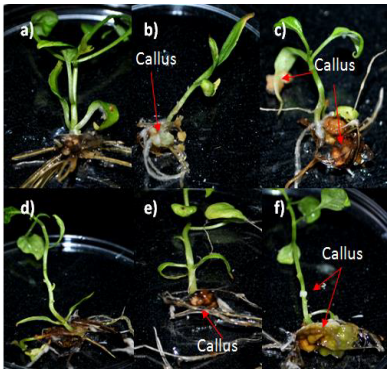

Figure 5. Callus formation of direct morphogenesis after 2 months in *C. annuum* **a)** absent **b)** moderate **c)** abundant and C. frutescens **d**) absent e) moderate **f)** abundant.

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in both *Capsicum* spp. hence the optimum level of BA concentration had been achieved at lower concentration. Present of BA had shown significant affect in stimulating the cell division towards shooting in hairy roots induced explants, which had similar properties to non-transform explants [30-33]. However, the hairy roots induce cotyledon explants had faster shoots induction process compare to the non-transform cotyledon explants due to the presence of hairy roots that enhance a transportation system during mineral and hormone uptake for rapid cell division. In this research study, heights of regenerated plant were measured as the rate of morphogenesis. The result had showed that, continuous supplement of BA had cause positive regeneration towards the height of the plant **(Figures 7 and 8)** which was similar in cases of recent *Capsicum* plant studies [43-45]. In *C. annuum*, presence of 1.5 mg/L of BA had continuously increased the rate of morphogenesis with highest elevation of height (6.01 ± 0.24 cm) after 2 months **(Figure 7)**. High BA concentration had shown increased in height for *C. annuum* however further increased in BA with 2.0 mg/L had cause delay in morphogenesis rate with 4.46 ± 0.27 cm. In *C. frutescens*, lower concentration of BA (1.0 mg/L) was required to achieve highest rate of morphogenesis which was 9.2 ± 0.28 cm **(Figure 8).** While, further increased and decreased of BA concentration lead to reduce in height. In early two weeks, the morphogenesis of *C. frutescens* were occur at similar rate in 1.0, 1.5, 2.0 mg/L of BA concentration with height relatively at 5.29 ± 0.26, 5.44 ± 0.46, 5.01 ± 0.24 cm which showed that, the different concentration of BA did not enhance the early morphogenesis process. The different concentration of

Table 1. Callus formation during direct morphogenesis of *Capsicum* spp. in BA.

List species	BA (mg/L)	Day of callus induced	Callus induction rate
C. annuum	**Control**	-	-
	0.25	-	-
	0.5	-	-
	1	38	+
	1.5	30	+
	2	28	++
C. frutescens	**Control**	-	-
	0.25	-	-
	0.5	-	-
	1	-	-
	1.5	30	+
	2	25	+
*Sign of callus: (-) = absent, (+) = moderate, (++) = abundant			

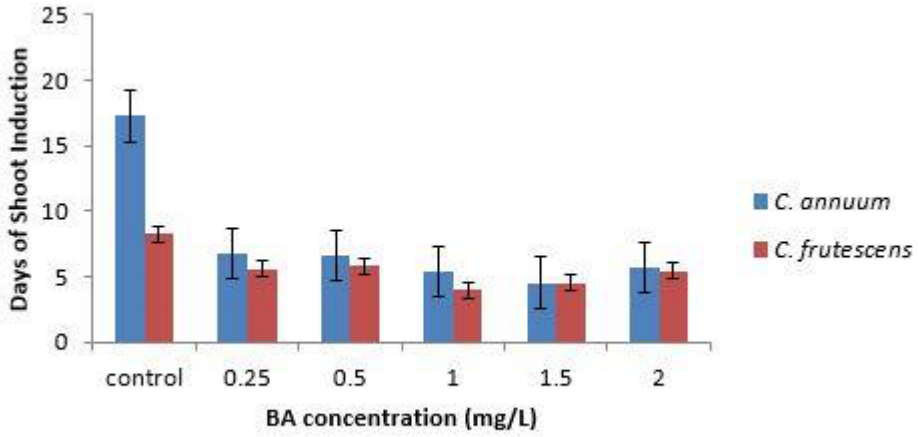

Figure 6. Days of shoot induction of direct morphogenesis of C. annuum and C. frutescens in different concentration of BA for 2 month.

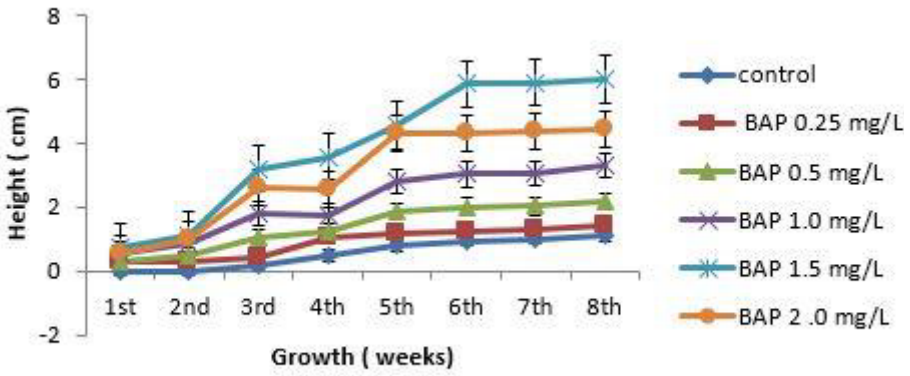

Figure 7. Grow rate of direct morphogenesis by hairy root induced explants of C. annuum in different concentration of BA for 2 month.

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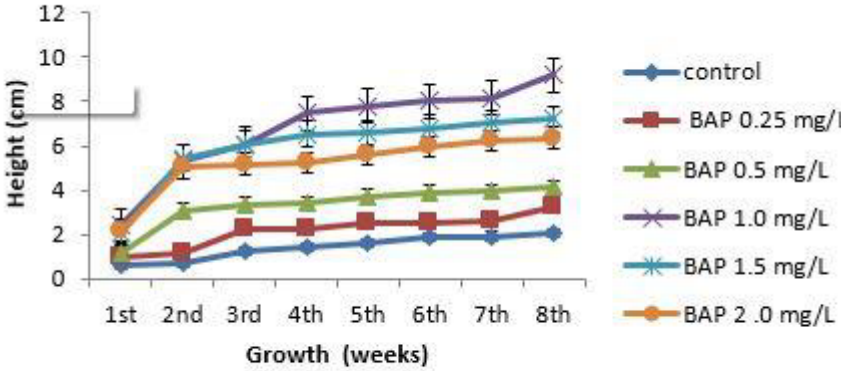

Figure 8. Grow rate of direct morphogenesis by hairy root induced explants of *C. frutescens* in different concentration of BA for 2 month.

BA had given significances differences in height for both species only after the one month of culturing. The overall height of the *C. frutescens* were higher than *C. annuum* after two month of growth period which about 2-3 cm differences. This was due to the rapid morphogenesis of other organ which was indicated by number of branches and leaves produced by *C. annuum* **(Table 2) (Figure 9)**. The number of branches and leaves had shown higher in 1.5 mg/L BA which most optimum for morphogenesis of *C. annuum.*

Table 2. Effect of different concentrations of BA during direct morphogenesis of *C. annuum* after two month.

BA (mg/L)	No. of leaves	No. of branches	Fresh weight (g)
control	1.95 ± 0.89^{d}	0.00 ± 0.00^{e}	0.25 ± 0.05^{f}
0.25	3.75 ± 0.91^{c}	0.00 ± 0.00^{e}	0.62 ± 0.09^{e}
0.5	5.45 ± 0.89^{b}	1.75 ± 0.91^{d}	1.01 ± 0.09^{d}
1	5.15 ± 0.75^{b}	2.70 ± 0.80^{c}	1.14 ± 0.08^{c}
1.5	9.65 ± 0.93^{a}	6.35 ± 0.93^{a}	1.68 ± 0.12^{a}
2	5.90 ± 0.79^{b}	4.15 ± 0.75^{b}	1.44 ± 0.13^{b}
*Means of the same superscripted letter did not differ significantly at P≤0.05			

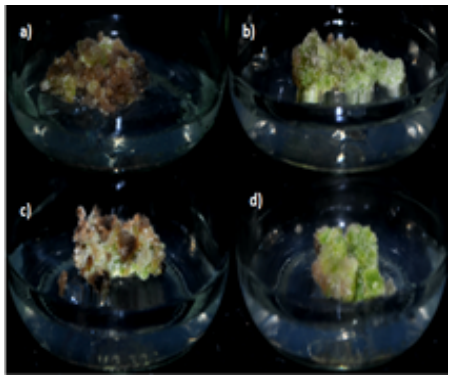

Figure 9. Recalcitrant of indirect morphogenesis for both *Capsicum* spp. in BA (2.0 mg/L) a) callus of *C. annuum* in 4 weeks b) green callus of *C. annuum* in 8 weeks a) callus of *C. frutescens* in 4 weeks b) green callus of *C. frutescens* in 8 weeks.

Different concentration of BA had high significant differences in resulting number of branches and leaves produced during regeneration. Absent and low concentration of BA (0.25 mg/L) had significantly affecting low number of leaves and lack branches compared to 1.5 mg/L of BA. However, 0.5, 1.0, and 2.0 mg/L of BA had showed no significant different in production of leaves but shows differences in number of branches. High rate of branches and leaves had affecting the fresh weight of the *C. annuum*. Although heights of *C. frutescens* were higher than *C. annuum*, however rate of morphogenesis of leaves and branches were relatively low **(Table 3).** In *C. frutescens*, absent and low concentration of BA (control, 0.25, 0.5 mg/L) were unsuccessful to regenerate any branches. While, the optimum levels of BA (1.0 mg/L) only succeed to regenerate up to 1-2 branches approximately. The number of *C. frutescens* leaves showed increment as BA increase to 1.0 mg/L (10.45 ± 0.76). However, the leaves reduce with the addition of high BA concentration which 1.5 and 2.0 mg/L. *C. frutescens* had higher morphogenesis rate towards the vertical apex in height compared to *C. annuum.* However, *C. annuum* had undergone morphogenesis by focusing towards the organs (horizontally) rated by high number of branches and leaves. Therefore, both of the species had similar rate of morphogenesis different optimum levels of BA (1.0 mg/L for *C. frutescens* and 1.5 mg/L for *C. annuum*). The similarities in the rate of morphogenesis of these two species were achievable due to hairy roots induced cotyledon explants. These explants not only gave a support during morphogenesis but presence of hairy roots able to enhanced nutrient transportation which clearly seen in control treatment that also induced

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shooting. Therefore, the used of hairy roots explants induced by *A. rhizogenes* possessed high rate of morphogenesis potential which also reduce possibility of recalcitrant of this plant. While, present of BA as shoot inducers were shown highly suitable with hairy roots explants due to lacked of necrosis and lower callus formations occur during morphogenesis. The presence of necrosis indicates cell death may cause by the toxicity of hormones due to over concentration or incompatibility. Each of plant hormones have different orientation of polarity in which it movement inside the cell also can describe their compatibility to use with plants [46-48]. The cell polarity its highly depends on capacity of ion hydrogen (H^+) and potassium ions (K^+) at membrane plasma where the nutrient transportation including hormone being maintained. However, the cell polarity was mainly marked by the types of explants that been used which in this case were hairy roots explants [49]. Therefore, increases concentration of BA had successfully showed increase of polarity towards hairy roots induced cotyledon explant that leads to regeneration of *C. frutescens and C. annuum.* However, high concentration of BA above the optimum level, which *C. frutescens* (1.0 mg/L) and *C. annuum* (1.5mg/L) had cause loss polarity between cell and hormone [49,50]. These can be seen via abundant formation of callus during morphogenesis **(Figures 3a and 3b)** and reduce rate of morphogenesis in term of height, number of leaves, branches, and fresh weight. Therefore, from the result had shown that, combination of optimum concentration BA and hairy root explant did created high synergistic effect to enhance rate of morphogenesis of *C. frutescens and C. annuum* [51].

Table 3. Effect of different concentrations of BA during direct morphogenesis of *C. frutescens* after two month.

BA mg/L	No. of leaves	No. of branches	Fresh weight (g)
control	2.75 ± 0.97^{e}	0.00 ± 0.00^{c}	0.30 ± 0.05^{e}
0.25	5.70 ± 0.98^{d}	0.00 ± 0.00^{c}	0.53 ± 0.17^{d}
0.5	7.90 ± 0.72^{bc}	0.00 ± 0.00^{c}	0.92 ± 0.22^{c}
1	10.45 ± 0.76^{a}	1.80 ± 0.89^{a}	1.63 ± 0.19^{a}
1.5	8.40 ± 0.94^{b}	1.85 ± 0.81^{a}	1.36 ± 0.26^{b}
2	7.35 ± 0.99^{c}	0.85 ± 0.98^{b}	1.23 ± 0.44^{b}

CONCLUSION

Hairy roots cotyledon explants induced by *A. rhizogenes* had shown suitability for direct morphogenesis of both *C. annuum* and *C. frutescens*. BA hormone had showed high synergistic effect with combination of hairy roots induced explants due to lack of necrosis and callus formation during direct morphogenesis. From the result, the optimum level of BA for C*. annuum* was 1.5 mg/L and *C. frutescens* was 1.0 mg/L in order to gain the highest morphogenesis rate. However, the morphology of both *C. annuum* and *C. frutescens* did not affected by the role of BA. An advance studies on other factors such as light, pH, temperature or other types of hormones that may affect the morphogenesis are highly recommended.

Author's Contributions and Conflict of Interest

NMS carried out overall research experiment, involved in design of the study, performed statistical analysis, acquisition and interpretation of data and prepared manuscript. NJS contributed in the overall design of research study, coordinate the research experiment and advising important intellectual content. Both authors do not have any conflict of interest in any possible reviewer or peer reviewer.

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