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IMPACT OF VARIOUS 2, 4-D CONCENTRATIONS AND DIFFERENT VITAMIN MIXTURES ON *in vitro* CULTURE OF COCONUT (*Cocos nucifera* L.) BY UTILIZING SEEDLINGS SHOOT TIP

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ABSTRACT

In this experiment, seedlings as sources for shoot tips was assembled from the coconut research institute (CRI) of Chinese Academy of tropical agricultural sciences germplasm, Wenchang, Hainan, China. The shoot tips were cultured on Y3 medium to study the impact of ten concentrations of 2, 4-D and three vitamin mixtures on survival, death and development % on *in vitro* culture establishment stage of coconut (*Cocos nucifera* L.). The obtained results revealed that all 2,4-D concentrations 4,7, 75, 100, 120 and 140 mg/L + any vitamin mixture did not give any development and high concentrations of 2, 4-D increased the death %. In any case, it can be concluded that the rate of differences in survival, death and development percentages exhibited by 2, 4-D concentrations was more pronounced than the analogous ones resulted by vitamin mixtures. At any rate, utilizing of (2, 4-D at concentration 25 mg/L+ vitamin mixture 1 or 2) exhibited the greatest values of survival percentage and reduced death percentage. Just as, increased the percentages of development in *in vitro* culture establishment stage of coconut (*Cocos nucifera* L.) through seedlings shoot tip.

Key words: coconut (*Cocos nucifera*), *in vitro*, shoot tips, 2, 4-D, vitamin mixtures, development

INTRODUCTION

Coconut (*Cocos nucifera* L.) is a high value perennial crop, developed in a few nations, contributing considerably to the improvement of nourishment, food security, employment and income generation. The business significance of coconut has been becoming all around quickly in the course of the most recent fifteen years, with high esteem items, for example, packed coconut water, virgin oil, coconut milk products, coco-biodiesel, fiber derivatives for the automobile industry and horticulture [Lao 2009, Roolant 2014].

The loss of coconut trees, because of seniority, phytosanitary threats, specifically phytoplasma diseases for example, lethal yellowing (LY), has prompted a decrease in coconut production [Batugal et al. 2005]. To keep up the developing markets and expanding interest for coconut items, replanting of the majority of the developed land worldwide just as the establishment of new cultivated land, is critically required. This massive errand can't be cultivated by customary proliferation through seed alone and would require *in vitro* propagation by somatic embryogenesis (SE) given its exceptional propagation capacity. This technique has been connected for the improvement of exceptionally proficient and financially practical conventions in

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various labs around the world. Different types of coconut explants have been tested, but most of the research carried out has focused on rachilla explants from immature inflorescence [Hornung and Verdeil 1999] and plumule explants from zygotic embryos [Sáenz et al. 1999].

What's more, *in vitro* culture techniques give a magnificent platform for the multiplication of stubborn species for example, coconut and thereby increasing the homogeneity of the plantations. The enthusiasm for utilizing tissue culture procedures for proliferation of coconut has been essentially expanded with the aim of developing a reliable clonal propagation method for both research purposes and commercial use. Recently, unfertilized ovary was identified as a more reliable explant for coconut micro-propagation through somatic embryogenesis [Perera et al. 2007]. Despite the fact that the quantity of clonal plants acquired still stays, low for business scale there is a green light with the nonstop creation of plantlets even at a low rate [Vidhanaarachchi et al. 2013].

One of the main considerations influencing the accomplishment of *in vitro* plant propagation is the decision of plant growth Regulators (PGRs). Subsequently, PGRs are frequently added to culture media to control diverse physiological responses in *in vitro*, prompting the creation of tissues (for example, callus) organs (for example, shoots and roots) or entire plants [Amoo and Staden 2013]. In any case, the engineered auxin 2, 4-D is accounted for to be the best plant growth regulator in inducing callogenesis in various explants of coconut [Verdeil et al. 1989, Ebert and Taylor 1990, Weerakoon 2004].

A few plants can synthesize the necessities of vitamins for their growth. Some vitamins are required for ordinary development and improvement of plants; plants as catalysts in different metabolic processes require them. They may go about as restricting components for cell development and separation when plant cells and tissues are grown in *in vitro* [Torres 1989].

In this way, the present work was locked in to develop a successful strategy in coconut tissue culture through examination the impact of different 2, 4-D concentrations and different vitamin mixtures to pick the best blend between 2, 4-D and vitamin mixture on *in vitro* culture establishment stage of coconut (*Cocos nucifera* L.) by utilizing seedlings as a source for shoot tip.

MATERIALS AND METHODS

Plant material. Seedlings as a source for shoot tips was assembled from the coconut research institute (CRI) of Chinese Academy of tropical agricultural sciences (CATAS) germplasm, Wenchang, Hainan, China. In this way, young seedlings of coconut were chosen to be sound and in great status.

Explant preparation. The external leaves and fibrous tissues surrounding the base of seedlings were removed cautiously until to get shoot tip. The shoot tip was cut into small sizes having height 8–10 cm, 3–4 cm diameter, and in a cylindrical shape. The shoot tips were washed with tap water for 15 min to remove any debris. To reduce browning, we have attempt numerous ways by utilizing antioxidants types and concentrations but the best way in this concern was as follow (the shoot tips were put into a chilled antioxidant solution (150 mg/L of citric acid and 100 mg/L of ascorbic acid) for 20–24 h at 4°C in a refrigerator under completely dark conditions. After taking out from the antioxidant solution, the shoot tips were washed with distilled water then dipped in 70% ethanol for 1 min. The shoot tips were surface sterilized by sodium hypochlorite solution at concentration 1.6% (30% v/v Clorox, commercial bleach), enhanced with two drops of Tween 20 per 100 mL of sterilization solution for 20 minutes shaken time to time. Then dipped in 70% ethanol for 1 min. Then shoot, tips were washed with sterile distilled water three times for 10 minutes with shaking to remove any trace of sterilization solution. In addition, shoot tip meristem will prepare again by removing the outer leaves from the inside parts and excising of shoot tip containing the apical meristem with 6–8 leaf primordia (2–3 cm length and 1.5 cm width) then soaking shoot tip meristem and rinse, it three times using autoclaved distilled water. Finally, cutting the meristem with shoot tips and some leaf primordia to 15–20 sections and put (3–5 sections) in one container jar (all the steps of preparation as shown in Figure 4).

Shoot tips *in vitro* culture and incubation conditions. The shoot tips were cultured on Y3 [Eeuwens 1976] medium under laminar flow cabinet aseptically with various 2, 4-D concentrations (10 concentrations) as appeared Table 1 with 40 g/L sucrose, gelled with 3.5 g/L gel rite as well as, active charcoal at 2.25 g/L

with every vitamins mixture which added to the culture medium as shown in Table 2 and the medium pH was adjusted to 5.75 with KOH before autoclaving for 20 min at 120°C. Then transferred to the incubation room which growth conditions were adjusted to with be completely dark conditions and 26 ±2°C of temperature.

Experiment lay out and statistical analysis. The experiment design was a factorial treatments arranged in a completely randomized design with three replications was employed in arranging the investigated treatments. Whereas, each replicate was represented by five jars to study the impact of ten concentrations of 2, 4-D and three vitamins mixtures as appeared in previously mentioned Tables (1 and 2). Though, ten 2, 4-D concentrations with the first vitamins mixture (Amount 1), ten 2, 4-D concentrations with the second vitamins mixture (Amount 2) and ten 2, 4-D concentrations with the third vitamins mixture (Amount 3). Anyhow, data of survival and death % as well as, development of explants due to 2, 4-D concentrations and vitamins mixtures were recorded at the end of five weeks from incubation and subjected to two-way analysis of variance (ANOVA) using SAS statistical software version 9.2 [SAS Inc. 2008]. Treatments means were separated by using Duncan's multiple range test (DMRT) [Hills and Thomas 1978].

RESULTS AND DISCUSSION

Specific and interaction effects of 2, 4-D concentrations and vitamin mixtures on survival % of coconut shoot tips *in vitro* culture at the end of 5 weeks of incubation

Specific effect. It is quite clear that all the survival percentage values responded specifically to each investigated factor. However, the rate of differences in survival percentage exhibited by 2, 4-D concentrations was more articulated than the comparable to ones come about by vitamin mixtures. Herein, the response of survival percentage to the specific effect of 2, 4-D concentrations pointed out clearly that the greatest values of survival % were significantly in closed relationship to 2, 4-D concentrations at 25 and 15 mg/L,

Table 1. Effect of 2, 4-D concentrations (mg/L) added to Y3 medium

Medium code	2, 4-D concentrations	Medium code	2, 4-D concentrations
Y3 1	4 mg/L	Y3 6	50 mg/L
Y3 2	7 mg/L	Y3 7	75 mg/L
Y3 3	10 mg/L	Y3 8	100 mg/L
Y3 4	15 mg/L	Y3 9	120 mg/L
Y3 5	25 mg/L	Y3 10	140 mg/L

Table 2. Vitamins mixtures added to Y3 medium volume used (6 ml/L)

Reactive	Amount 1 (mg/L)	Reactive	Amount 2 (mg/L)	Reactive	Amount 3 (mg/L)
Nicotinic acid	0.05	Nicotinic acid	0.1	Nicotinic acid	0.15
Pyridoxine (HCl, VB6)	0.05	Pyridoxine (HCl, VB6)	0.1	Pyridoxine (HCl, VB6)	0.15
Thiamine (HCl, VB1)	0.05	Thiamine (HCl, VB1)	0.1	Thiamine (HCl, VB1)	0.15
Glycine	1	Glycine	2	Glycine	3
Folic acid	0.05	Folic acid	1	Folic acid	2
Biotin	0.05	Biotin	1	Biotin	2

respectively. Moreover, 2, 4-D concentrations at 10 mg/L ranked statistically 2nd, followed by its 4 and 7 mg/L. However, the lightest survival percentages were always in concomitant to the highest 2, 4-D concentrations above than 75 mg/L.

As for the specific effect of vitamin mixtures, obtained data as shown in Table 3 revealed that the rate of response was relatively not so pronounced to that previously discussed with 2, 4-D concentrations. Hence, the most elevated survival percentages was significant exhibited by (vitamin mixture 1).

Interaction effect. Concerning the interaction effect of various combinations between two considered factors i.e., 2, 4-D concentrations and vitamin mix-

Table 3. Specific and interaction effects of 2, 4-D concentrations and vitamin mixtures on survival % of coconut shoot tips i*n vitro* culture at the end of 5 weeks of incubation

Vitamin mixture / 2, 4-D concentration	Survival ratio (%)			
	Vit. mixture 1	Vit. mixture 2	Vit. mixture 3	Mean
Y_3 1 + 4 mg/L	66.67 de	66.67 de	53.33 ef	62.22 C
Y_3 2 + 7 mg/L	73.33 cd	53.33 ef	46.67 fg	57.78 C
Y_3 3 + 10 mg/L	80.00 bcd	73.33 cd	66.67 de	73.33 B
Y_3 4 + 15 mg/L	93.33 ab	86.67 abc	80.00 bcd	86.67 A
Y_3 5 + 25 mg/L	100.00 a	93.33 ab	73.33 cd	88.89 A
Y_3 6 + 50 mg/L	46.67 fg	33.33 gh	26.67 hi	35.56 D
Y_3 7 + 75 mg/L	26.67 hi	26.67 hi	20.00 hij	24.44 E
Y_3 8 + 100 mg/L	6.67 jh	13.33 ijh	6.67 jh	8.89 F
Y_3 9 + 120 mg/L	0.00 k	6.37 jh	0.00 k	2.22 F
Y_3 10 + 140 mg/L	0.00 k	0.00 k	0.00 k	0.00 F
Mean	49.33 A	45.33 A	37.33 B	

Means within a column followed by the same letter/-s are not significantly different at p = 0.05 based on DMRT

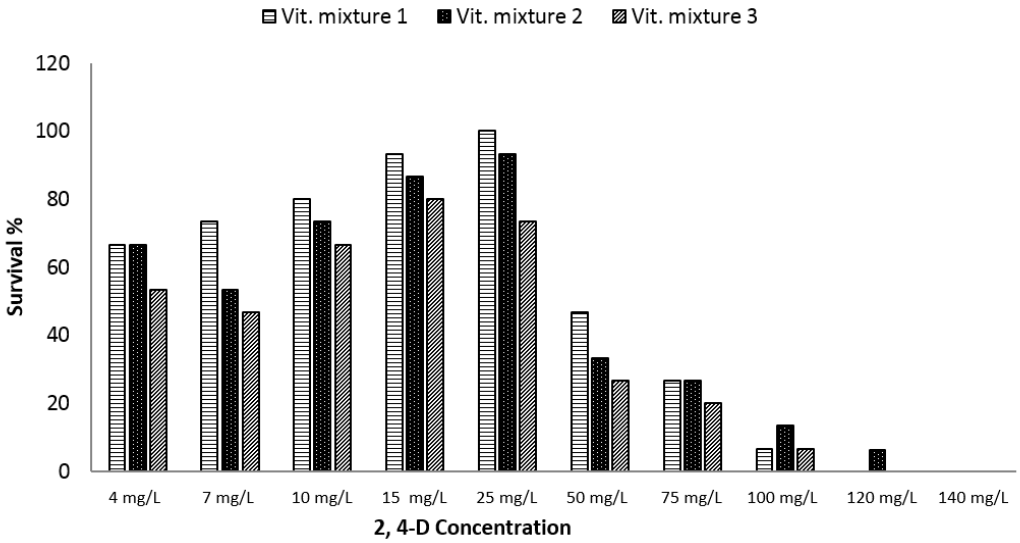

Fig. 1. Specific and interaction effects of 2, 4-D concentrations and vitamin mixtures on survival % of coconut shoot tips *in vitro* culture at the end of 5 weeks of incubation

Table 4. Specific and interaction effects of 2, 4-D concentrations and vitamin mixtures on death % of coconut shoot tips *in vitro* culture at the end of 5 weeks of incubation

Vitamin mixture / 2, 4-D concentration	Death %			
	Vit. mixture 1	Vit. mixture 2	Vit. mixture 3	Mean
Y_3 1 + 4 mg/L	33.33 gh	33.33 gh	46.67 fg	37.78 D
Y_3 2 + 7 mg/L	26.67 hi	46.67 fg	53.33 ef	42.22 D
Y_3 3 + 10 mg/L	20.00 hij	26.67 hi	33.33 gh	26.67 E
Y_3 4 + 15 mg/L	6.67 jk	13.33 ijk	20.00 hij	13.33 F
Y_3 5 + 25 mg/L	0.00 k	6.67 de	26.67 hi	11.11 F
Y_3 6 + 50 mg/L	53.33 ef	66.67 de	73.33 cd	64.44 C
Y_3 7 + 75 mg/L	73.33 cd	73.33 cd	80.00 bcd	75.56 B
Y_3 8 + 100 mg/L	93.33 ab	86.67 abc	93.33 ab	91.11 A
Y_3 9 + 120 mg/L	100.00 a	93.33 ab	100.00 a	97.78 A
Y_3 10 + 140 mg/L	100.00 a	100.00 a	100.00 a	100.00 A
Mean	50.67 B	54.67 B	62.67 A	

Means within a column followed by the same letter/-s are not significantly different at p = 0.05 based on DMRT

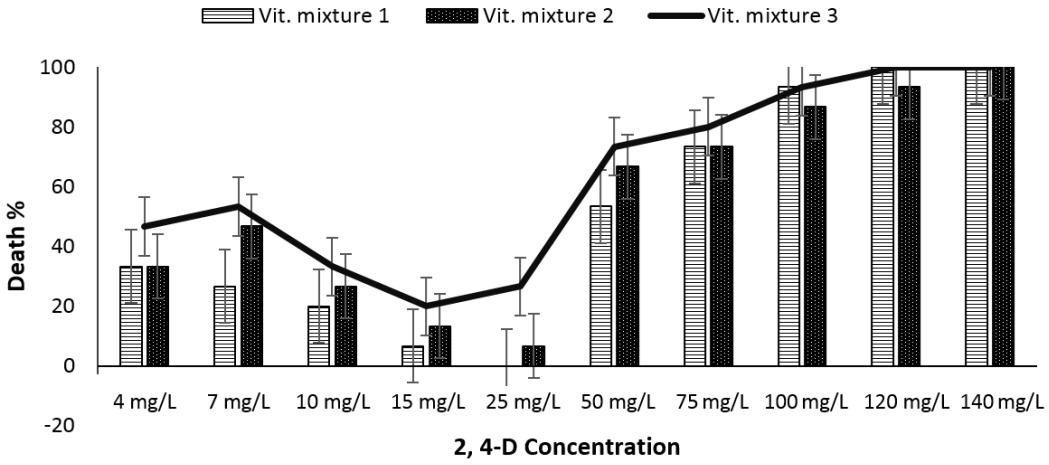

Fig. 2. Specific and interaction effects of 2, 4-D concentrations and vitamin mixtures on death ratio (%) of coconut shoot tips in vitro culture at the end of 5 weeks of incubation

tures tabulated data in Table 3, Figures 1 and 5 uncovered clearly that each investigated factor reflected directly its specific effect on interaction effect of their combinations. Thus, the combinations of (25 mg/L 2, 4-D + vitamin mixture 1) exhibited statistically the greatest values of survival percentage. On the contrary, the least values of the of survival % were always in closed relationship to these combinations of (140 mg/L 2, 4-D + all the vitamin mixtures). What's more, other combinations were in-between the previously mentioned extremes.

Specific and interaction effects of 2, 4-D concentrations and vitamin mixtures on death ratio (%) of coconut shoot tips *in vitro* culture at the end of 5 weeks of incubation

Specific effect. As for the specific effect of 2, 4-D concentrations, data obtained revealed that all high concentrations (more than 50 to 140 mg/L) increased significantly the values of death %. Moreover, the most effective in death percentage coupled with its highest concentration of 2, 4-D (from 75 to 140 mg/L).

Concerning the specific effect of vitamin mixture, obtained results displayed that differences in death % values were not so pronounced and the response to vitamin mixtures concentration didn't follow a firm trend similar to that previously detected with 2, 4-D concentrations. Anyhow, the third vitamin mixture resulted in a highest increase in death percentage. Such increase didn't reach level of significance with (vitamin mixture 1 and vitamin mixture 2).

Interaction effect. With regard to the interaction effect of various combinations of two studied factors (2, 4-D concentration × vitamin mixture) on the death % are presented in Table 4, Figures 2 and 5. Different combinations varied obviously pertaining their effect on death %. Herein, the most effective combinations which increased death % were generally in closed relationship to those between three concentrations of 2, 4-D (100, 120 and 140 mg/L) from one hand and any vitamin mixture from the other. However, on the other hand, 25 mg/L of 2, 4-D combined with both vitamin mixture (1&2) were statistically the most effective in reducing the death %. In addition, other combinations were in between the aforesaid two extremes.

Specific and interaction effects of 2, 4-D concentrations and vitamin mixtures on development % of coconut shoot tips *in vitro* culture at the end of 5 weeks of incubation

Specific effect. Regarding to the specific effect of differential investigated factors. It is quite clear that development percentage response to different factor. Moreover, the superiority of 2, 4-D concentrations could be explained on the base of their physiological role. However, 2, 4-D at 25 mg/L gave the greatest values of development %, Meanwhile, 2, 4-D at 75, 100, 120 and 140 mg/L did not give any development.

Concerning the specific effect of vitamin mixture, obtained results in Table 5 displayed that differences in development percentages were not so pronounced and the response to vitamin mixtures concentration

Table 5. Specific and interaction effects of 2, 4-D concentrations and vitamin mixtures on development ratio (%) of coconut shoot tips *in vitro* culture at the end of 5 weeks of incubation

Vitamin mixture / 2, 4-D concentration	Development ratio (%)			
	Vit. mixture 1	Vit. mixture 2	Vit. mixture 3	Mean
Y_3 1 + 4 mg/L	0.00 f	0.00 f	0.00 f	0.00 C
Y_3 2 + 7 mg/L	0.00 f	0.00 f	0.00 f	0.00 C
Y_3 3 + 10 mg/L	13.33 de	20.00 cd	13.33 de	15.56 B
Y_3 4 + 15 mg/L	20.00 cd	20.00 cd	13.33 de	17.78 B
Y_3 5 + 25 mg/L	66.67 a	60.00 a	46.67 b	57.78 A
Y_3 6 + 50 mg/L	6.67 ef	26.67 c	20.00 cd	17.78 B
Y_3 7 + 75 mg/L	0.00 f	0.00 f	0.00 f	0.00 C
Y_3 8 + 100 mg/L	0.00 f	0.00 f	0.00 f	0.00 C
Y_3 9 + 120 mg/L	0.00 f	0.00 f	0.00 f	0.00 C
Y_3 10 + 140 mg/L	0.00 f	0.00 f	0.00 f	0.00 C
Mean	10.67 AB	12.67 A	9.33 B	

Means within a column followed by the same letter/-s are not significantly different at p = 0.05 based on DMRT

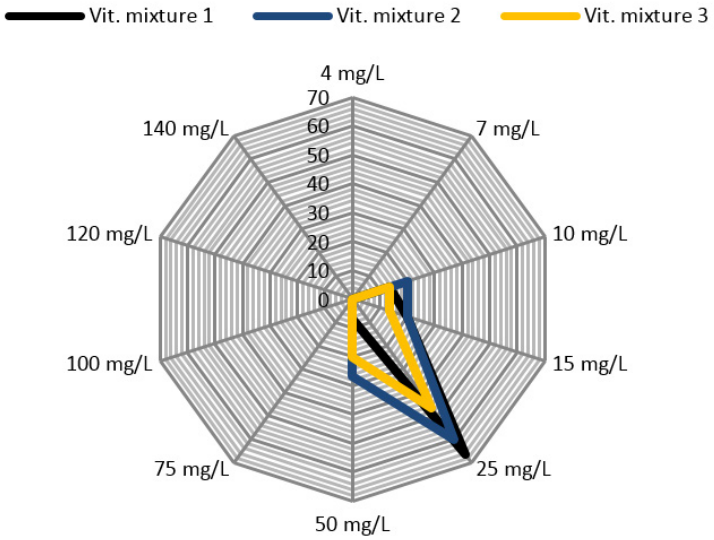

Fig. 3. Specific and interaction effects of 2, 4-D concentrations and vitamin mixtures on development % of coconut shoot tips *in vitro* culture at the end of 5 weeks of incubation

specially vitamin mixture 1 and 2. Anyhow, the second vitamin mixture resulted in a highest increase in development percentage.

Interaction effect. Table 5, Figures 3 and 5 display obviously that the highest development % were significantly in concomitant to the combination of (2, 4-D at 25 mg/L+ vitamin mixture 2). The obtained results revealed that all, 2, 4-D at 4,7, 75, 100, 120 and 140 mg/L + any vitamin mixture did not gave any development.

DISCUSSIONS

To accomplish effective callus generation in coconut explants, it is additionally basic to characterize the most appropriate blend of 2, 4-D and activated charcoal, two of the essential components in the callus induction medium. The valuable impacts of activated charcoal are credited to its adsorption of phenols and other growth inhibitory substances. In any case, it likewise adsorbs 2, 4-D present in the culture medium, leading to undefined conditions with regard to available 2, 4-D concentration in the medium [Ebert and Taylor 1990, Verdeil et al. 1994, Córdova-Lara et al. 2016]. This thus could prompt variable tissue performance and non-reproducible results.

The phase of cell multiplication, identified with the affectability to the 2, 4-D of foliar tissues of date palm, occurs as of the fourth seven days of culture [San´e et al. 2006] and prompts the formation of the primary calluses after the 28 days of culture. The callogenesis stage requires the utilization of elevated exogenous auxin levels in many species [Fehér et al. 2003]. The impact of the 2, 4-D amid cellular dedifferentiation firmly associated with an expansion in endogenous AIA in carrot tissues [Michalczuk et al. 1992]. Undoubtedly, results acquired in *Medicago sativa* demonstrated that concentrations in endogenous AIA increased considerably amid the initial 3 days of culture in the presence of optimal concentrations of 2, 4-D [Pasternak et al. 2000]. The accumulation of endogenous AIA in tissues under 2, 4-D treatment would beat the origin of the totipotency of somatic cells in *Zea mays* and thusly of their ability to be directed towards embryogenesis [Jemenez and Bangerth 2001].

Plant regulators or PGR in like manner impact the age and activity of the [Lebon et al. 2004] plant growth regulators accept a basic occupation in the improvement and formation of discretionary metabolites in the plant tissue and cells culture. For example, type and gathering of the auxin or cytokinin and auxin-cytokinin ratio have significant effects on both

the growth and aggregation of secondary metabolite in plants [Ozyigit 2008].

Meziani et al. [2015] noticed that a PGR-free medium accentuated tissue browning (95%) in "Mejhoul", however the least tissue browning (20%) was found in a medium including 0.2 mg/L NOAA, 0.2 mg/L IAA, 0.4 mg/L Kin and 0.4 mg/L 2iP, when the shoot tips were used as explants. In date palm, browning would thus the capacity to be constrained by varying the levels of PGRs in the medium. However, Calli induced from media that had lower levels of 2, 4-D were creamish. Moreover, the colour intensified as the concentration of 2, 4-D in the induction medium increased. In a related study in chickpea [Zaman et al. 2010].

The vitamins most utilized in the cell and tissue culture media incorporate thiamin (B1), nicotinic acid and pyridoxine (B6). Thiamin is essentially required by all cells for growth [Ohira et al. 1976]. Thamin utilized at concentrations extending from 0.1 to 10 mg/L. Nicotinic acid and pyridoxine, anyway

Fig. 4. Explant preparation (seedlings as sources for shoot tips all the steps from the field to get shoot tip for *in vitro* culture)

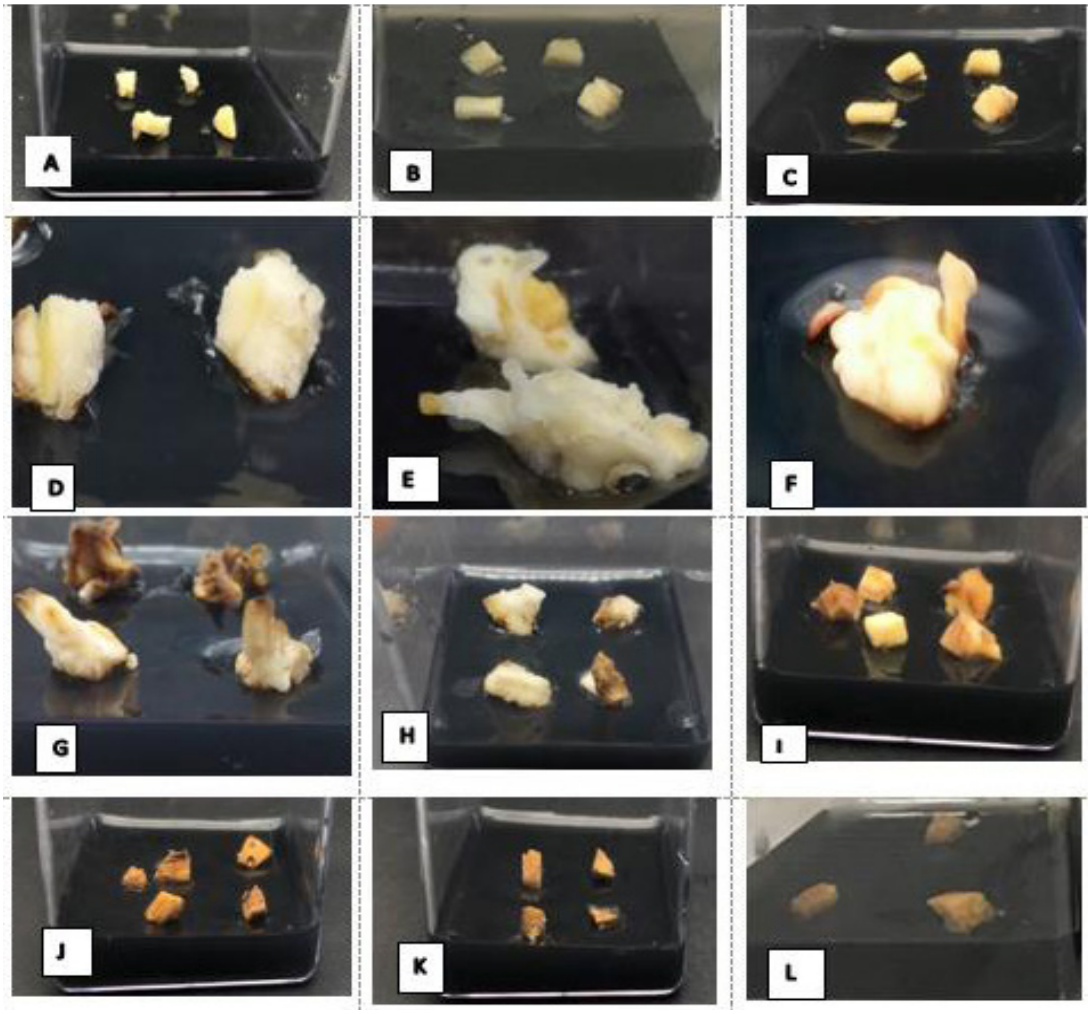

Fig. 5. Interaction effects of 2, 4-D concentrations and vitamin mixtures on coconut shoot tips *in vitro* culture at the end of 5 weeks of incubation. Whereas, A = 4 mg/L 2, 4-D + vitamin mixture 1; B = 7 mg/L 2, 4-D + vitamin mixture 1; C = 10 mg/L 2, 4-D + vitamin mixture 1; D = 15 mg/L 2, 4-D + vitamin mixture 1; E = 25 mg/L 2, 4-D + vitamin mixture 1; F = 25 mg/L 2, 4-D + vitamin mixture 2, G = 50 mg/L 2, 4-D + vitamin mixture 1; H = 50 mg/L 2, 4-D + vitamin mixture 2; I = 75 mg/L 2, 4-D + vitamin mixture 1; J = 100 mg/L 2, 4-D + vitamin mixture 1; K = 120 mg/L 2, 4-D + vitamin mixture 1 and L = 140 mg/L 2, 4-D + vitamin mixture 1

not basic for cell growth of many species, they are frequently added to culture WH media [White 1943]. Nicotinic acid is utilized at a concentration range 0.1–5 mg/L and pyridoxine is utilized at 0.1–10 mg/L. Other vitamins for example, biotin, folic acid, ascorbic acid, pantothenic acid, tocopherol (vitamin E), riboflavin, p-amino-benzoic acid are utilized in some cell culture media in any case; they are not growth limiting factors. It was prescribed that vitamins ought to be added to culture media just when the concentration of thiamin underneath the ideal dimension or when the cells are required to be developed at low population densities [Vasil and Thorpe 1998]. In spite of it is not a vitamin but a carbohydrate, myo-inositol is included little amounts to invigorate cell growth of most plant species.

CONCLUSION

Definitively, from the acquired results, it can be concluded that the rate of differences in survival, death and development percentages exhibited by 2, 4-D concentrations was more articulated than the practically equivalent to ones come about by various vitamin mixtures. At any rate, utilizing of (2, 4-D at concentration 25 mg/L + vitamin mixture 1 or 2) demonstrated the best estimations of survival percentage and reduced death percentage. As well as, increased the percentages of development in *in vitro* culture establishment stage of coconut (*Cocos nucifera* L.) through shoot tip and gave initial callus.

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