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Total steroids production from date palm callus under heavy metals stress

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Date palm is considered one of the most important commercial crops in the Arab worlds. Biotechnological applications of plant cell cultures presents the most updated reviews on current techniques in plant culture in the field, rapid propagation of date palm through tissue culture is the most promising technique for production of sufficient planting materials (off shoots) and obtaining high quality In vitro plant cell cultures have potential for commercial production of secondary metabolites. Date palm tissues produced steroids which have important medicinal value. There are few studies about the enhancement and increasing the production of date palm plant cell of these important secondary metabolites. This study was conducted to stimulate total steroid content production from date palm friable callus tissue by adding some heavy metals as abiotic elicitor stress (Cadmium chloride ($CdCl_2$) and Aluminum chloride ($AlCl_3$)) to growth nutrient medium during callus production stage. It have been tested two concentrations for each type from heavy metals under investigation, this concentrations are 0.05 and 0.1 g L^{-1} $CdCl_2$ and 0.03 and 0.06 g $L^{-1}AlCl_3$ to induce total steroids production from callus of date palm. Callus fresh weight (g), differentiation percentage, average number of somatic embryo and total steroids (mg/g dw) were determined after 8 weeks from its treated. Data showed that, $CdCl_2$ treatments promoted and increased friable callus tissue and total steroids production while, $AlCl_3$ treatments developed and induced differentiation of friable callus explant of date palm cv. Sewi. In recent years, secondary metabolites of date palm have received special attention given their health-benefit claims and potential use in the booming industries of functional foods and nutraceuticals. A number of studies are under way to unveil more properties and in establishing procedures and protocols to economically and efficiently incorporate these date-derived products in the diet.

Keywords: Date Palm, Biotechnological applications, Secondary metabolites, Elicitor Heavy metals, Cadmium chloride, Aluminum chloride, Total steroids

INTRODUCTION

Date palm tree (*Phoenix dactylifera* L.) is considered as one of the oldest and main staple and ancient crops in Southwest Asia and North Africa. (Abdel-Aal,2011) Dates are a monocotyledonous tree belongs to Arecaceae family. The importance of date palm tree is referred to the high nutrition value of its fruits and the great yields of the whole tree. It is considered to be the most substantial fruit crops, which played valuable roles in the economy, society, and environment for local populations especially in the Middle East and North African countries (Ajungla et al.,2009) Higher plants such as date palm accumulate a wide range of different chemicals in their tissue. These can conveniently be divided into two types of compounds: products of primary plant metabolism such as proteins, fats and carbohydrates, which guarantee the primary functions of growth and development; and

products of secondary metabolism such as phenolics. Phenolic compounds are widely distributed in the plant kingdom. These organic compounds are not directly involved in primary metabolic processes of growth and development but are important constituents of resistance/tolerance to stress. These include lignins and other phenolics, which strengthen mechanically the cell walls while tannins, flavonoids, and some simple phenolics serve as defenses against herbivores, pathogens, ultraviolet radiation and other abiotic stress(Al-Alawi et al.,2017)

Date palm as a source of secondary metabolites, date fruit is a rich source of sugar, nutrients and pharmaceutical secondary metabolites such as phenolic, citric acid, oxytetracycline and ethanol as well as essential oils, polyphenols and dietary fibers. The phenolic compounds, hydroxycinnamates, Gallic acid derivatives, monohydroxybenzoic acids, flavones and anthocyanins are widely distributed in date palm (Bailey AE, 2005)

Steroid belong to a large group of compounds known as terpenoids or isopernoids. Nothing may well be suggested concerning drugs which are utilized in the curing of sterility except date palm pollen grains, which have been recognized by the Egyptian and Arabs to be nutritive and used as antisterility agent. Cholesterol and coprostanol are the animal sterols, while, B-sitosterol, campestral, stigmasterol, ergosterol and brassicasterol are the principal plant sterols(Biglari et al.,2008)

Plants and plant cells in vitro show physiological and morphological responses to microbial, physical or chemical factors, which are known as elicitors. Since the secondary metabolites protect plants from the environmental changes, the way to induce their synthesis is to apply unfavorable factors, i.e., simulate pathogen attack, herbivores, heavy metals, etc. Elicitation is a process of induced or enhanced synthesis of secondary metabolites by the plants to ensure their survival, persistence, and competitiveness. Biotic and abiotic elicitors are used to stimulate secondary metabolite product formation in plant cell cultures(Cetin1 et al.,2014)

The molecules that stimulate the production of secondary metabolites are termed as elicitors. Both biotic and abiotic elicitors induce product accumulation not only in intact plants or plant organs but also in plant cell cultures as a result of their defensive, protective or offensive reactions (Chakravarty and Srivastava 1992, El-Dawayati et al.,2012).

Heavy metals have one of the main abiotic stress agents for secondary metabolites production. The evolving commercial importance of the secondary metabolites has in recent years a great interest, in secondary metabolism, and particularly in the possibility to alter the production of bioactive plant metabolites by means of cell culture technology. The principle advantage of this technology is that it may provide continuous, reliable source of plant pharmaceuticals and could be used for the large-scale culture of plant cells from which these metabolites can be extracted. Advances in the area of cell cultures for the production of medicinal compound have made possible the production of a wide variety of pharmaceuticals like alkaloids, terpenoids, steroids, saponins, phenolics, flavanoids, and amino acids. Successful attempts to produce some of these valuable pharmaceuticals in relatively large quantities by cell cultures are illustrated(El Hadrami et al.,2011)

In vitro plant cell cultures have potential for commercial production of secondary metabolites. Date palm tissues produced steroids which have important medicinal value(El-Sharabasy and El-Dawayati , 2017). Our study aimed to induce and increase total steroid production from date palm callus by adding some heavy metals, Cadmium chloride and Aluminum chloride as abiotic elicitors stress to basal nutrient medium during callus production stage of date palm cv. Sewi.

MATERIALS AND METHODS

This work was carried out at The Central Lab of Date Palm Researches and development, Giza, Egypt to induce steroid production from date palm callus by adding some heavy metals such as Cadmium chloride and Aluminum chloride as abiotic elicitors stress to the basal nutrient medium during callus production stage.

Establishment of explants material

Sterilized shoot tip from young suckers of date palm cv. Sewi grown in El Giza governorate were used as sources for callus induction. The explants were cultured on Murashige and Skoog (MS) basal nutrient medium (Gantait et al.,2018)] contained 10.0 mgl^{-1} 2,4–D dichlorophenoxy acetic acid (2,4–D) and 3 mgl^{-1} 2-isopentenyl adenine (2iP) (Gronsv, 2005). The explants subcultures to fresh medium at each 6 weeks interval five times at least and maintain under total darkness at 27±1 °C incubation condition to produce friable callus which use in this study.

Effect of heavy metals as abiotic elicitors on callus growth and total steroids production

In this study 1.0 g friable callus tissue was used as explants materials. The explants cultured on MS basal nutrient medium supplemented with 30 g L^{-1} sucrose, 40 mg L^{-1} adenine – sulfate, 200 mg L^{-1} glutamine, 100 mg L^{-1} myo-inositol, 0.1 mg L^{-1} biotin, 170 mg L^{-1} KH_2PO_4,0.1 mg L^{-1} thiamine HCL 0.5 mg L^{-1} pyridoxine, 0.5 mg L^{-1} nicotinic acid, 0.1 mg L^{-1} NAA and 6 g L^{-1} agar, 0.01 mg L^{-1} pyruvic acid (Hamel et al.,1998) as a control treatment and different concentrations of Cadmium chloride ($CdCl_2$) 0.05 and 0.1 g L^{-1} and Aluminum chloride ($AlCl_3$) 0.03 and 0.06 g L^{-1} separately. Each treatment included 3 replicates each replicate included 3 small jars (150 ml) and each jars containing 1.0 g callus tissue. The culture jars were incubated under total darkness at 27±1 ° C to induce steroid production.

Data were recorded after eight weeks on growth and development of friable callus tissue (fresh weight (g) differentiate percentage and average number of somatic embryo) and total steroids (mg/g dw).

Determination of total steroids (mg/g dry weight)

Test solution preparation: - 0.5 g weight of embryogenic callus sample is dried in an oven at 75 _C for 48 h. dried embryogenic callus sample is placed in a clean flask, with addition of 100 mL of 5% potassium hydroxide solution in alcohol (90% v/v) and are heated on a water bath at 50_C to smooth reflux for 2 hours, then are cooled for 5 min, then the flask contents are transferred to a separator funnel. The residual contents of flask were washed for two times, firstly with 100 mL water followed by 100 mL diethyl ether then the washings were transferred into the same separator funnel and they are shacked altogether slowly by hand for 3 min. To separate the formed layer the aqueous phase was removed from separator funnel. This layer was washed in a separator funnel four times with 100 mL diethyl ether then, is placed in a clean flask. The received ethereal extracts are washed with three successive portions of 40 mL water (shaking was gently to avoid emulsions), 40 mL 5% w/v hydrochloric acid, and 40 mL 3% w/v potassium hydroxide aqueous solution. Successive portions of 40 mL water (each wash) are edited until the washings become neutral to phenolphthalein solution (2 drops 1% phenolphthalein in 70% ethanol and 2 N NaOH until rose color is stable). One drop of 0.1 N HCl is added to sample and rapidly mix until the rose color disappears. 100 mg anhydrous sodium sulfate powder is added to the sample with well shacking, then the mixture is filtered through folded Whatman filter paper. The resulted solution is evaporated in water bath at 50 _C until fully dry. 100 mL glacial acetic acid is added to the residue with stirring for 30 min in small glass bowl.

Test solution: - 2ml of previous resulted solution is transferred to a 20 mL volumetric flask, and dilute to 20 mL with glacial acetic acid.

The reference solution preparation:- 40 mg β-sitosterol is dissolved in 100 mL glacial acetic acid then 5 mL of this solution is taken then diluted to 50 mL with glacial acetic acid .

The deniges reagent preparation: - This reagent is consist of mixing of two solutions (solution A) is prepared by adding 100 mL sulfuric acid to 50 mL glacial acetic acid. .(solution B) is prepared by dissolving 5g mercury oxide (HgO2) and 20 mL sulfuric acid into 100 mL water. 100 mL of solution (A) is added to 1 mL of solution (B), then are mixed and filtered through a sintered glass filter (grade G4) before use.

Finally 5 mL of Deniges reagent mixture solutions is added to test tube filled with 1 mL (Test solution) and 1 mL (Reference solution) for evaluation of β-sitosterol amount.

The blank is carried out by 1 mL glacial acetic acid instead of the sample in a test tube. Both tubes are lifted on the stand under the dark for 15 min. The absorbance is read using a spectrophotometer at 510 nm against the blank reading. The amount of steroids is calculated as β-sitosterol from a standard curve prepared by dissolving 40 mg of β-sitosterol in 10 mL glacial acetic acid. Series of standards are prepared as 5, 10, 20, and 40 mg/100 mL, respectively; 1 mL of each is mixed with 5 mL Deniges reagent, and read at 510 nm against the blank. The absorbance of each concentration is plotted against the absorbance obtained from the standard curve.

Total steroids were calculated as β-sitosterol and determined by spectrophotometer according the methods described by (Hamelet al.,1998),(Ibrahim et al.,2017)and(Iwai et al.,2003) as follows.

Statistical analysis

The factorial design in completely randomized arrangement was used and data were subjected to analysis of variance. Separation of means among treatments was determined using L.S.D

test at 5% probability level according to (Kamada et al.,1993)

RESULTS AND DISCUSSION

The results presented the impact of heavy metals as abiotic elicitors on the morphological growth, development of friable callus tissue and steroids accumulation of date palm cv. Sewi after eight weeks from culturing

Effect of heavy metals as abiotic elicitors on callus growth and development

The morphological changes observed during this study show influence $CdCl_2$ and $AlCl_3$ on growth and development of friable callus tissue explant of date palm cv. Sewi.

Fig. 1 illustrates effect of different concentrations $CdCl_2$ and $AlCl_3$ on friable callus fresh weight (g). There are high significant differences among all treatments in this experiment. $CdCl_2$ at 0.1 g L^{-1} achieved maximum value of friable callus weight (15.56 g) after 8 weeks of culture, this results are not agree with Shekhawa et al who stated that Callus *Brassica juncea*, which have been exposed to different concentrations of cadmium (5, 25, 50, 100, 150, and 200 µM), showed reduction in growth rate. Moreover, reddish brown patches were observed in calli exposed to 100, 150, and 200 µM CdCl2, respectively (Kamada et al.,1998). Control treatment achieved a minimum value of friable callus weight (5.33 g) while $AlCl_3$ treatments (0.03 and 0.06 g L^{-1}) recorded (10.33 and 7.16 g) of callus weight. These results are line with Suman and Kalpana(Kiyosue et al., 1990)declared that Seeds of *Eleusine coracana* were inoculated on callus induction medium with varied Cd levels (100µM, 300 µM and 500 µM) Calli were formed in treatments with 100 µM concentration of Cd .While there was no callus formation in treatments with 300 and 500 µM concentrations of Cadmium. Also In *Eleusine coracana* callus induction obtained in the medium containing 100 µM concentration of Pb and Ni but there was better growth obtained in Ni containing medium than Pb **(**Kokate et al.,2002)In addition Chakravarty and Srivastava (Krishania and Agarwal 2012)studied the effects of six heavy metals, aluminum (Al), cadmium (Cd), copper (Cu), nickel (Ni), lead (Pb), and zinc (Zn) were studied in the growth rate of callus tissues in vitro were compared to ascertain the concentrations that can either support plant growth or cause lethality. Highest toxicity to the plant system was observed from the effects of Pb both at high and low concentrations whereas Zn was the least toxic. The clastogenic effects of Al, Cd, Cu, and Ni were dependent on concentration and length of treatment. Cu and Zn showed less severe cytotoxic damages than Al, Cd, Pb, and Ni.

On the other said, Fig. 2 shows effect of different concentrations $CdCl_2$ and $AlCl_3$ on differentiation percentage of friable callus explant of date palm cv. Sewi after eight weeks from culturing on treatments under investigation. All concentrations of $CdCl_2$ and control treatment not stimulate differentiate of friable callus where recorded 0.0 % of differentiation friable callus explant. While all concentrations of $AlCl_3$ (0.03 and 0.06 g L^{-1}) promoted friable callus growth and developed it to embryonic callus cells (pro embryo) where achieved high differentiation percentage of embryonic callus (33.33 and 44.44%)

Fig. 3 appears effect of different concentrations $CdCl_2$ and $AlCl_3$ on average number of somatic embryos. $CdCl_2$ treatments (0.05 and 0.1 g L^{-1}) and control treatment not induce somatic embryosgenesis production where, it recorded 0.0 embryo/explant. Further, $AlCl_3$ at 0.03 g L^{-1} produced 0.88 embryo/explant and $AlCl_3$ at 0.06 g L^{-1} produced 1.22 embryo/explant.

Clearly, $CdCl_2$ treatments promoted and increased callus production (Fig. 4) while, $AlCl_3$ treatments developed and induced differentiation of friable callus explant of date palm cv. Sewi (Fig. 5). These results agree with (Iwai et al.,2003) (Meets et al.,2005) who declared that Somatic embryogenesis is induced in Arabidopsis with heavy metal ion, moreover, certain stresses may induce somatic embryogenesis in various plant species. The fact indicates that stress induces a common reaction, and the reaction is related to the plant somatic cell de-differentiation and re-differentiation to somatic embryogenesis. Probably, stress treatment induces expression of a factor that controls the start of somatic embryogenesis, through the common reaction. In carrot (Michalak A, 2006., Mohan JS, 2013, Mulabagal et al.,2004) declared that somatic embryos could be induced by heavy metal ion stress (Cd^{2+}, Fe^{2+}, etc.). Further, in *Rauvolfia serpentine*, maximum callus biomass was observed in 0.15 mM $AlCl_3$ amended medium with a highest fresh weight of 1.24 g and dry weight of 0.14 g at day 6. To understand the role of $AlCl_3$ in plant defense mechanism, various antioxidant enzymes i.e. superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) activities were assayed in in vitro cultivated calli

with or without $AlCl_3$ treatment. The above enzymes activities increased linearly with increasing $AlCl_3$ level, i.e. 5.10 enzyme unit (EU) min^{-1} mg^{-1} protein SOD, 3.93 EU min^{-1} mg^{-1} protein CAT and 0.83 EU min^{-1} mg^{-1} protein APX, all being maximum at 0.20 mM treatment (Murashige and Skoog, 1962)Also our results showed high browning appearance on friable callus tissue of dale palm cv. Sewi when it was treated by all concentrations of $CdCl_2$ and $AlCl_3$ (data un tabulated).

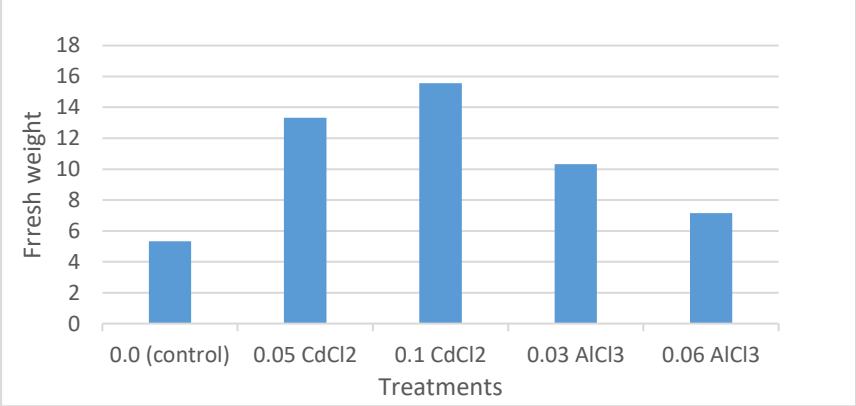

Figure.1 Effect of different concentrations of Cadmium chloride ($CdCl_2$) and Aluminum chloride ($AlCl_3$) on Fresh weight (g) of friable callus tissue of date palm cv. Sewi after 8 weeks

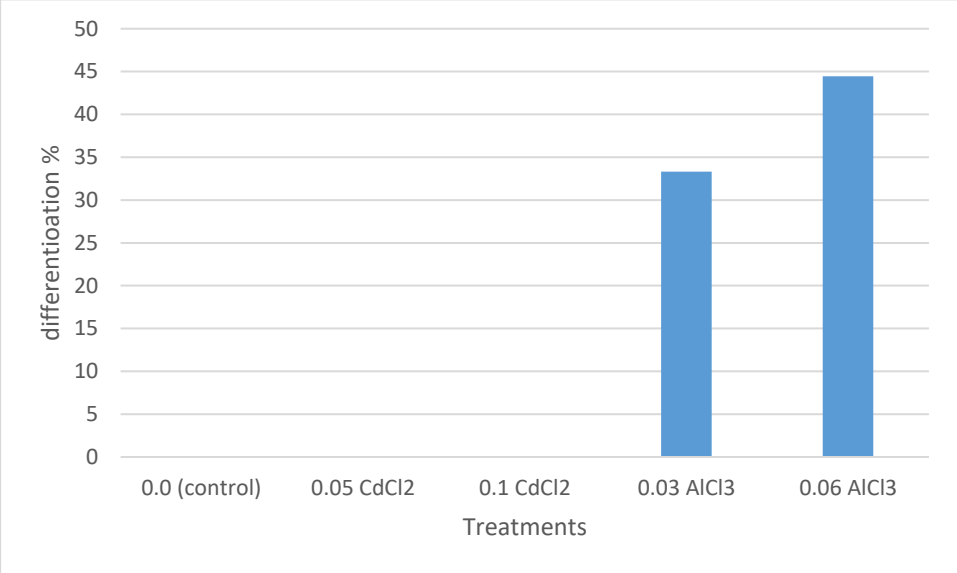

Figure.2 Effect of different concentrations of cadmium chloride($CdCl_2$) and aluminum chloride ($AlCl_3$) on differentiation percentage of callus friable tissue of date palm cv.Sewi after 8 weeks

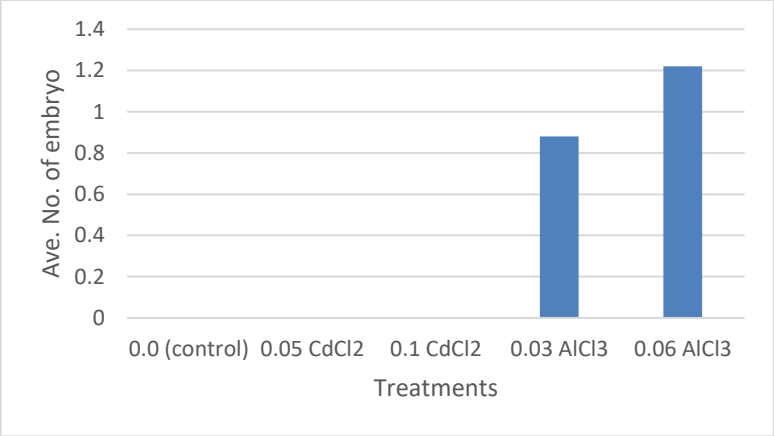

Figure.3 Effect of different concentrations of cadmium chloride($CdCl_2$) and aluminum chloride ($AlCl_3$) on average number of somatic embryo of date palm cv.Sewi after 8 weeks

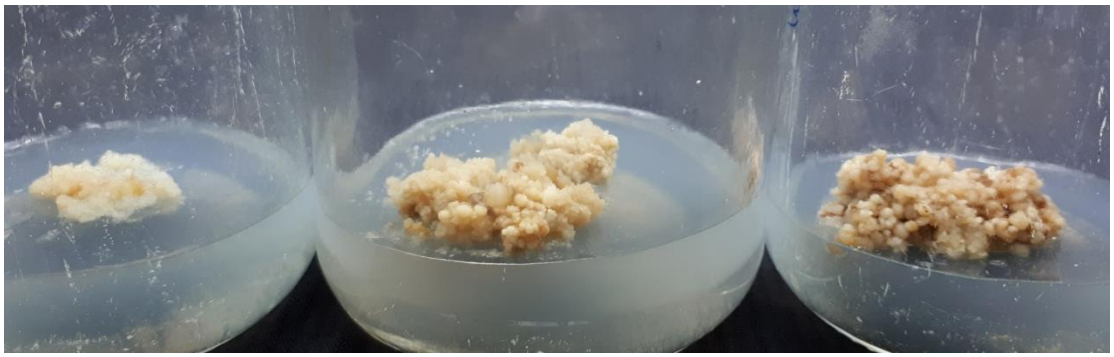

Figure. 4 The effect of different concentrations of $CdCl_2$ on fresh weight, differentiation % and somatic embryo number of friable callus tissue explant of date palm after 8 weeks

Figure. 5 The effect of different concentrations of $AlCl_3$ on fresh weight, differentiation % and somatic embryo number of friable callus tissue explant of date palm after 8 weeks

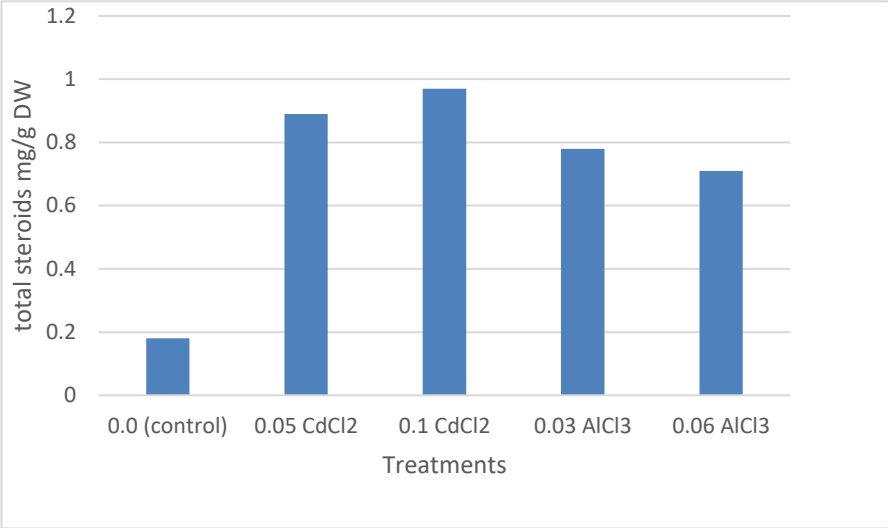

Figure. 6 Effect of different concentrations of cadmium chloride($CdCl_2$) and aluminum chloride ($AlCl_3$) on total steroids (β-sitosterol mg/g d.w.) of date palm cv.Sewi after 8 weeks

These results agree with Michalak **(**Namdeo , 2007.)who indicated that, there have been many reports of induced accumulation of phenolic compounds and peroxidase activity in plants treated with high concentrations of heavy metals.

Determination of total steroids (mg/g dry weight)

Regarding, the impact of different concentrations $CdCl_2$ and $AlCl_3$ on total steroids production from embryonic callus of date palm cv. Sewi, data in Fig. 6 clearly showed that, adding different concentrations of $CdCl_2$ (0.05 and 0.1 g L^{-1}) and $AlCl_3$ (0.03 and 0.06 g L^{-1}) in the basal nutrient medium of embryonic callus, stimulated in an increased total steroids content in embryonic callus culture compared with control treatment with high significant differences among them. Moreover, $CdCl_2$ at 0.1 g L^{-1} achieved the maximum value of total steroid content (0.97mg/g dw) followed by $CdCl_2$ at 0.05 g L^{-1} recorded 0.89 mg/g dw. As well as, $AlCl_3$ at 0.03 0.1 g L^{-1} generated 0.78 mg/g dw of total steroids content and $AlCl_3$ at 0.06 g L^{-1} generated 0.71 mg/g dw of total steroid content without significant differences in between. On the other hand, the control treatment recorded the lowest value of total steroids content (0.18mg/g dw).

Secondary metabolite contents were increased by cadmium chloride application, these increases might be explained by hypothesizing that Cd act as a stress factor on grape cell cultures which stimulate and alter the pathways responsible for phenolics and tocopherol biyosynthesis (Nasim and Dahir 2010).Cadmium causes oxidative stress probably through indirect mechanisms such as interaction with the antioxida-tive defense, disruption of the electron transport chain or n. The activation of lipoxygenase, an enzyme that stimulates lipid peroxidation, has been reported after cadmium exposure (Snedecor and.Cochran 1972). Our results confirmed that, there was the increase in steroids content in the callus culture after treated with 0.33 g/L $AlCl_3$ and those results was agreed with Ajungla et al .,(Shekhawat et al.,2010)which say that, there was the increase in the hyoscyamine and scopolamine content in root culture in the medium containing $AlCl_3$ (25-250Mm), these results indicated that $AlCl_3$ can stimulate the production of tropane alkaloids. Increased contents of hyoscyamine and scopolamine were also obtained in hairy root cultures of Brugmansia candida after treatment with $AlCl_3$ (Smetanska , 2008). The reason for this might be that most of the genes up-regulated by $AlCl_3$ homologies with those related to pathogenesis, suggesting aluminum may act as an elicitor (Spollansky et al.,2000)The exposure to heavy metals leads to accumulation of harmful reactive oxygen species (ROS). Plants counteract

the toxic effects of heavy metal stress by activating certain metabolic activities and physiological odifications (Suman and Kalpana , 2013)these protect the plant against free radicals and prevent damage to plant molecules such as lipids, proteins and nucleic acids. Some of the strategies include accumulation of plant secondary metabolites such as antioxidant enzymes, proline, glutathione and phenolic and flavonoids compounds (Zafar et al.,2017)When the treatment is used in high concentrations, the secondary metabolite accumulation decreases (Pharco. Assay of total steroids (calculated as B-sitosterol). 1993) Thus, might be explained decrease total steroids when friable callus explant treated with high concentration of $AlCl_3$.Moreover, research is needed to clarify the mechanism by which heavy metals induce responses that result in enhanced secondary metabolite production.

CONCLUSION

Our study aimed to induce and increase total steroid production from date palm callus by adding some heavy metals Cadmium chloride and Aluminum chloride as abiotic elicitors stress to basal nutrient medium during callus production stage of date palm cv. Sewi. It is seems to be that increasing in friable callus tissue was companion with increased in total steroids control which were affected by $CdCl_2$ where somatic embryogenesis differentiation was suppressed. As well as $AlCl_3$ treatments promoted friable callus growth and developed it to embryonic callus cells (pro embryo) where achieved high differentiation percentage of embryonic callus and increasing number of somatic embryos differentiated. Also our results showed high browning appearance on friable callus tissue of dale palm cv. Sewi when it was treated by all concentrations of heavy metals. In recent years, secondary metabolites of date palm have received special attention given their health-benefit claims and potential use in the booming industries of functional foods and nutraceuticals (Zayed , 2017)A number of studies are under way to unveil more properties and in establishing procedures and protocols to economically and efficiently incorporate these date-derived products in the diet (Al-Alawi et al.,2017)

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

ZEZ designed and performed the experiments and also wrote the manuscript. MME performed steroid estimation experiments and data analysis. SFE collected materials and reviewed the manuscript. All authors read and approved the final version.

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