Effect of biotic factors stresses on vinblastine and vincristine production from callus of *Catharanthus roseus*

Kareem T Khashan
Faculty of science
University of Kufa

Mohommed A. Husain
Collage of agriculture
University of Al- Qasim

Abstract:

This experiment was conducted in collage of Science labs, Kufa University, carried out during 2013-2014 to study many experiments inducing callus tissues from leaves *(Catharanthus roseus* L) *in vitro* using MS medium supplemented with Dichlorophenoxiactic acid (2,4-D) at different concentration (0.5, 1, 1.5) mg/L with the interaction of (BA) benzyl adenine at concentrations of (0.5, 1, 1.5) mg/L to obtain the optimum combination for callus induction which then use it in the following experiments and investigate the effect of biotic factors (fungi ,yeast and bacteria) on vinblastine and vincristine production from callus tissue , and leaves of mother plant. This study include determination the catalase activity , and total proline as responsible to the variety of the stresses in callus as well as quantities and qualities determination of vinblastine and vincristine TLC(Thin layer chromatography and HPLC(high performance liquid chromatography) .Finally, comparison study were made between *in vitro* and *in vivo* grown plant .

Results in this study revealed that the highest fresh weight of callus was for (1mg/L 2,4-D + 1mg/L BA) combination treatment .Also, Results showed an increasing in proline content as responsible to biotic factors .The highest value was for yeast extract agar treatment 2g / L .However, The highest catalase enzyme activity was for *Trichoderma harzianum* treatment ; ***Azotobacter chroococcum*** bacteria ,followed by Yeast extract agar treatment with significant different compared with control treatment. The result showed a significant increasing ($P< 0.05$) of these alkaloids production and the superiority of vinblastine and vincristine content in Callus than the content in leaves of mother plant .

Key Words: Culture media, callus, CAT, vinblastine , vincristine. Biotic factors

ISSN 2072-3875

تأثير اجهادات العوامل الاحيائية على انتاج الفنبلاستين والفنكرستين من كالس نبات عين البزون .

كريم طالب خشان محمد عبد الحسن حسين

الخلاصة :
تم اجراء التجربة في مختبرات كلية العلوم / جامعة الكوفة خلال الفترة 2014 لدراسة مجموعة من التجارب والتي اشتملت على استحثاث انسجت الكالس من اوراق نبات عين البزون(.*Catharanthus roseus* L) بزراعتها على وسط MS (Murashinge and Skooge, 1962) مزود بمنظم النمو ال (2,4-D) -2,4 dichlorophenoxiactic acid وبتراكيز مختلفة (0.5,1,1.5) ملغم/لتر (طور النشوء) وبالتداخل مع منظم النمو Benzyl adenine (BA) وبتراكيز مختلفة (0.5,1,1.5) ملغم/ لتر للحصول على افضل التراكيز المستخدمة في استحثاث الكالس اعتمادا على مقدار الوزن الطري . بعد ذلك اعيدت زراعة وزن محدد من انسجة الكالس على وسط MS مضافا اليها العوامل الاحيائية (الفطريات، الخميرة والبكتيريا) لدراسة تاثيرها على كمية انتاج المركبات القلويدية من خلال كالس النبات المدروس. تم تحديد الوزن الطري والجاف للكالس في جميع المعاملات . استخدمت تقنية ال HPLC للكشف عن نوعية وكمية مركب vinblastine وvincristine في اوراق وكالس النبات من خلال استخدام محلول قياسي للمركبين ومقارنة كميتهما في انسجة الكالس لجميع المعملات مع كميتهما في الاوراق . تم قياس بعض المؤشرات الكيموحيوية في انسجة الكالس تحت الاجهاد الحيوي وبدون اجهاد مثل فعالية انزيم الكتليز وكمية الحامض الاميني البرولين. أشارت النتائج بان افضل توليفة من الهرمونات النباتية في استحثاث انسجة الكالس هي 2,4-D بتركيزmg/L +BA 1mg/L ولوحظ زيادة في فعالية انزيم الكتليز في انسجة الكالس كاستجابة للاجهادات الحيوي مقارنة مع انسجه الكالس غير معرضه للاجهاد وكانت اعلى قيمة سجلت في معاملة مستخلص الخميره(2)غم/لتر اما بخصوص فعالية انزيم الكتاليز فقد سجلت اعلى قيمه في معاملة الفطر *Trichoderma harzianum* ثم المعاملة بالبكتريا وبعدها المعاملة بمستخلص الخميرة مع وجود فروقات معنويه مقارنة بمعاملة السيطرة. واظهرت النتائج وجود زيادة معنوية ($P<0.05$) لمركبي الفنكرستين والفنبلاستين في انسجة الكالس المعرض للاجهادات الاحيائية عند مقارنتها مع كمية المركب في الاوراق وكانت اعلى قيمه عند المعاملة 2غم /لتر مستخلص الخميرة .

الكلمات المفتاحيه: وسط الMS ، كالس، كتليز، برولين، مركب الفنبلاستين والفنكرستين. العوامل الاحيائية.

Introduction :

For a long time medicinal plants considered an important source of medicine value (Cragg and Newman ,2005) . They are economically important to human due to their multiple applications ,such as Pharmaceuticals ,flavors ,fragrance ,insecticides ,dyes ,food additives and toxins and important either to protect these plants against microorganisms and animals or to enhance the competition with other plants in a particular habitat also provide protection against UV radiation and different forms of stresses (Zhao *et al*., 2005) . Biosyntheses is of these compounds although controlled genetically and several enzymes that contributed in these reactions are also affected strongly by environmental factors and soil conditions (Al- Hatamy , 2006).Secondary metabolite can be classified according to the basis of chemical structure to three main groups , terpene, phenolic and alkaloids compounds (Fumihiko *et al* ,2000).

ISSN 2072-3875

Catharanthus roseus (L.) G.Don. belongs *Apocynaceae* family extensively investigated medicinal plants produce more than 200 important compounds (mainly terpene indole alkaloids) the anticancer ,Vinblastine and vincristine in low amounts and depends on the physiological and development stage of the plant and in most cases has not been economically feasible(Zhuo and Verpoorte ,2007).These chemicals are often less, produced in very small amounts(less than 1% of total carbon)(Cragg and Newman,2005). The high cost of isolating the drugs has caused researchers to use plant tissues culture technique to increase the alkaloids contents of ***C.roseus*** in vitro. The valuable antineoplastic alkaloids vinblastine and vincristine extracted from ***C. roseus*** have been long target of researchers trying to increase their production. In vitro aseptic culture of cells, tissues, organs under controlled nutritional and environmental conditions, often to produce and increase the important compounds as a secondary metabolites among many and various applications were obtained from tissues culture such as the production of varieties tolerant. Several researchers studied the effect of variety combinations of growth regulators 2,4-D, 2,4-D + Kin , NAA and NAA + BA in the nutrition medium to induce callus from stems , leaves , seeds , found that the treatment contains 1 mg/L 2,4-D + 1mg/L Kin is the best Callus were produced alkaloids from petiole of ***C. roseus***. (Taha *et al*,2008). Also, alkaloid pathways are not only regulated tissues specifically and developmentally (Facchini 2001),but are also affected by external biotic factors (Zhao *et al* 2005).Suspension cultures of *C. roseus*were elicited with fungal cell wall fragments of *Aspergillus niger*, *Fusarium moniliforme,* and *Trichoderma viride* on ajmalicine accumulation were studied. A higher concentration of elicitor extract responded positively to *C. roseus* suspension cultures. Ajmalicine accumulation increased by about 3-fold when cells were treated with A. *niger*, *F. moniliforme*, and *T. viride*. (Ajay Namdeo,2002). *A. chroococcum* bacteria strains added to nutrient media effected on callus growth of two *Beta vulgaris* L. cultivars were investigated, resulted in the highest callus mass, , glutamine synthetase(GS) andglutamate dehydrogenase(GDH) activity (Kumar *et al* ., 2007). Yeast extract is also applied to enhancement of secondary metabolite production in vitro. Al-Mafargi , (2010) refer that the effect of yeast extract on secondary products from rosemary callus culture was determined by comparing the product percentage after four weeks of exposure , to that non-elicited controls. The major component produced from callus treated with 1 g/L of yeast extract agar on cineol , camphor , borneol, terpineol, verbinone and bornyl acetate were increased as compared to control . To investigate the effect of biotic factors (fungi, yeast and bacteria) stress on vinblastine and vincristine production from callus as a stress factors. The aims

ISSN 2072-3875

of this study is to examine the effect of different growth regulators on callus induction from ***C.roseus*** leaves explants and enhanced vinblastine and vincristine production in callus tissue. Although investigation of current study is to select callus lines tolerant to biotic stress of biotic factors (fungi ,yeast and bacteria) and characterize callus lines with respect to growth, alkaloids content, osmolytes accumulation and activity of catalase antioxidant enzymes and compare with its content in leaves mother plant.

Materials and methods:

preparation of leaves and callus tissue for alkaloids extraction

C. roseus plants were collected from greenhouses of Agriculture college /AlQasim university in January 2014. leaves of plant was extracted by Soxhelet (Continuous extraction) for 24 hours using ethanol 80 % , Extraction was concentrated to dryness by rotary evaporation(Harborne,1984).However, Alkaloid compounds were extracted from callus tissues , according to AL-Hattab *et al*, (2000) by 500 mg from dry callus was crushed well in mortar casserole ,after drying wet callus in oven on 40 C for 24 hr with ethanol 80 % and ether 20 % at 1/4(v/v) ratio.

Detection of vinblastine and vincristine alkaloids by (TLC)

Silica gel plates (precoated) were used in 0.25 mm thickness and dimensions of 20 * 20 cm and placed in electric oven at a temperature of 120 C and left to cool , then put raw leaves , callus extraction spots and standard solutions for alkaloids vinblastine and vincristine each alone by drops using (ammonium hydroxide: water: acetone) 25 % at ratio(3:7:90) used as separate solution for alkaloids separation ,the separate spots were limited by eyes and by UV-light at wave length 254 nm. For detection of separated alkaloid compounds by estimate R*f* according to Harbone, (1984).

Quantity determination of vinblastine and vincristine compounds in raw leaves extraction by (HPLC)

HPLC good were :Nucleosil 5 C18 ,column (250 * 4.6 mm ,5 µm),Samples were eluted with (methanol/ acetonitrile/ 0.025 M ammonium acetate/triethylamine by 15:40:45:0.1 , volume),at 1 ml/min. ,monitored at 280 nm(Zhao *et al* ., 2001).

The standard solutions for calibration curves

The solutions were prepared by dissolve 1mg from pure vincristine in 9 ml HPLC grade water to obtain on 100 µg/ml and by serial dilutions to obtain on 10 , 1 µg/ml and by the same method for vinblastine sulfates. Compounds concentrations in samples were estimated as the following :

ISSN 2072-3875

$$\text{Alkaloid} = \frac{\text{Relative area for sample}}{\text{Standard Relative area for standard}} \times \text{alkaloid concentration in sample}$$

Medium Preparation and explants sterilization :

The leaves of ***C. roseus*** were washed by running tap water and liquid soap several times to remove dust and dirt and transferred to laminar air flow cabinet for surface sterilization by submerged in ethanol 70 % with shaking for 30 second then washed three times by distilled water and were sterilized by sodium hypochlorite 6 % for 20 minutes then washed three times by distilled water and prepared for culture. MS medium was used for callus induction in the form of packets with weight of 4.4 grams, which is used for preparation of a liter from tissues culture medium .Sucrose was added at 30 g/L and agar at 8 g / L , with pH of 5.8 ± 2 then transferred to autoclave for sterilization on 121 C and pressure 1.04 kg/cm² for 20 minutes and left till cool .

Callus inducing phase :

The sterilize leaves were cut to parts with approximately 1cm² area ,cultured on MS medium supplemented with plant growth regulators 2,4-D in concentrations (0.5 ,1, 1.5) mg/l and BA in concentrations (0.5 ,1 , 1.5) mg/l as nine interaction combinations then transferred to growth chamber under suitable conditions on 25±2 C and photoperiod 16/8 hr. light/darkness on 1000 lux light intensity , for get the best combination to form callus. After 45 days ,fresh weight was taken for limit the best combination to use it in the following experiments to reach the best treatment for inducing callus and use it in accumulation phase for next experiments . **Accumulation phase**

Preparation of biotic factors

***Trichoderma harzianum* fungus and fungus extraction factors**

The fungus *T.harzianum* used as biotic factor using two experiments

First*: T. harzianum*was activated on a new Bitridish transferred to suitable conditions for growth in incubator at 25 C , after 7 days the growth was complete and by cork borer 3 disks were taken from exit edge of growth putting in flask contains 250 ml broth **.D** ,closed well ,transferred to incubator for 7 days , then the fungal extraction was taken by Buokhner funnel with filtration paper Whattman No 1 and vacuum pump . 0.1ml from fungal extraction v/v was added to the MS medium with the best treatment of plant growth regulators (with notice agar quantity) , putting in the sterile glasses tubes and exacted pH on 5.8 ± 2 and transferred to autoclave for sterilization and left till cool transferred to cabinet then cultured 250 mg fresh weight from callus on MS medium for increase the metabolic compounds , transferred to growth chamber at 25 C and 16/8 hr photoperiod . Second : single spore method , 250 mg

ISSN 2072-3875

callus harvested and sub cultured on MS medium with the best treatment of plant growth regulators and inoculated directly by single spore in the glass tubes ,closed well and transferred to growth chamber at 25 C and 16/8 hr. photoperiod till the fungus was covered the callus (Eufrocinio *et al*., 2002).

***Azotobacter chroococcum* bacteria extraction factor :**

A. chroococcum was grow on free nitrogen medium (sucrose mineral salt solution) 250 ml in flask, , which maintains on Ashbys medium transferred to the flask by the needle and were kept in incubator for suitable conditions , then after 2 days extraction was added at 10 ml/L to flask which contains MS medium with the best treatment of plant growth regulators and autoclaved at 121 C and a pressure at 1.04 kg/cm² for 20 min after transferred to sterile glasses tubes and exacted pH on 5.8 ± 2 then left to cool ,placed in laminar cabinet for cultured 250 mg callus on the medium, for study the effect of this bacteria on accumulation of alkaloid compounds transferred to growth chamber at 25 C and 16/8 hr. photoperiod.

Yeast culture(Yeast extract agar factor):

Yeast extract agar was added at concentration (1 , 2 , 3) g / L to MS medium and putting in sterile glass tubes transferred to autoclave for sterilization after exacted pH on 5.8±2 , left till cool and transferred to cabinet for cultured 250 mg callus on the medium, to study effect of yeast extract agar on accumulation of alkaloid compounds transferred to growth chamber at 25 C and 16/8 hr. photoperiod.

Catalase enzyme activity determination

Catalase enzyme activity determination in leaves and callus extraction in inducing phase and callus extraction in accumulation phase for all treatments as the following 1 g of samples were crushed with 10 ml buffer phosphate by adding 0.3 g PVP using ceramic mortar under ice ,the extraction was filtrated by filtration papers, Centrifuges at 10000 rpm for 10 min. under 4 C .Then 20 ml from enzymatic extraction was taken and added for it 1 ml hydrogen peroxide 30 % ,incubated for 1 min then the readings were taking for determination the catalase activity at 240 nm by UV-spectrophotometer.

$$\text{Catalase activity (unit)} = \frac{\text{Abs/min} * \text{Reaction volume}}{0.001}$$

Abs = the difference between the first absorption and the second.

Min = reaction time. 2.4 ml = reaction volume . 0.001 = constant.

ISSN 2072-3875

Proline determination :

According to Bates *et al* (1973) method ,proline was determined in leaves and callus extraction in inducing phase and accumulation phase. The red toluene layer was reading by UV-spectrophotometer at 520 nm while blank sample consist of 5ml toluene ,then wave length for variety concentrations of proline were measured for standard solution .Proline concentration calculate According to Bates et al (1973).

Statistical analysis :

Statistical analysis was according to factorial complete random design for interaction treatments and just complete random design for others .10 replicates are used for each treatment and treatments medium were tested by the least significant different(L.S.D) at improbability level 5 % according to system of SPSS (Levesque , 2007).

Results and discussion :

Detection of vinblastine and vincristine by (TLC)

The result explained the presence of six compounds when examined by eyes and by UV- light ray ,at 254 nm wavelength .R*f* values on TLC plates are (25,30,60,75,85,95) in leaves extraction and (25,35,65,75,85,95) in raw callus extraction. The results showed that R*f* for vinblastine and vincristine (95 , 85) respectively are equal to two of raw and leaves extraction and they are another values with different values of R*f* to vinblastine and vincristine and not identified due to the unavailability standard solutions (Al-Hatamy.,2006 ; Smesim , 2012). Dragendorff reagent gave positive detection(Orange spot) on silica gel plates.

Effect of plant hormone combinations on callus fresh weight :

Results in the table(1) the combination treatments between auxin and cytokinin is very important to obtain of the highest quantities from fresh weight of callus to use in the new experiments. The results referred to the highest fresh weight (3.276) g was for (1mg/L 2,4-D + 1mg/L BA) combination treatment fig(2) ,followed by (2.606) g for (0.5 mg/L 2,4-D+ 0.5 mg/L BA) and (2.498) g for (1.5mg/L 2,4-D + 1.5 mg/L BA) combination treatment respectively with significant different among all treatments . Carew and Krueger;(1977) indicated that 2,4-D and BA combinations are important to increase fresh weight and inducing callus in ***C. roseus***. Ethbaieb (2010) indicated that the best combination for callus inducing in *C.* roseus was 2 mg/l auxin with 2 mg /L cytokinin . Auxins effects directly of cell expansion through increase some enzymes activities which response of wall elasticity and increase permeability (George *et al* ; 2008).The cytokinins are important for cell division due to induce biosynthesis of some important proteins and for RNA formation(Davies , 2004).

ISSN 2072-3875

Table (1) : Effect of hormone combinations on Fresh weight of callus.

BA(mg/L) / 2,4-D(mg/L)	0.5	1.0	1.5	Means
0.5	2.606	1.250	1.100	1.632
1.0	1.447	3.267	1.384	2.032
1.5	1.872	1.553	2.498	1.973
	1.975	2.023	1.660	1.886

LSD for auxins 0.146 : cytokinin 0.146 : interaction : 0.084

Fig(2): Callus induced from leaves of *C.roseus* grown in (1mg/L)2,4-D and BA(1mg/L)

Effects of biotic factors on callus fresh(g) and dry weight (mg)

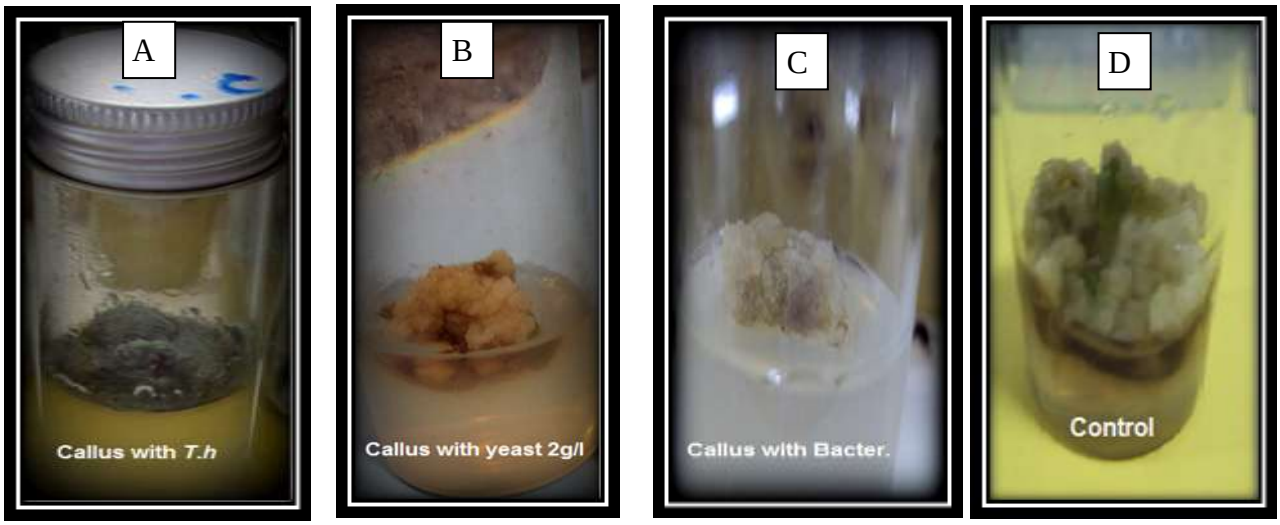
The results shown in table(2) referred that the addition of biotic factors cause decrease in fresh weight and dry weight compared with control treatment (without any factor). ***T.harzianum*** treatment gave the lowest fresh weight (0.615) g fig(3A) compared with control treatment(3.267) g fig(3D) followed by yeast extract agar treatment 3 g / L (0.949) g fig(3B) with significant different compared with control treatment and without significant different compared with 2 g /L extract yeast agar treatment followed by Bacteria treatment ***(A .chroococcum***) gave (1.642) g fig(3C). The results indicated in the same table that the lowest dry weight was for ***T.harzianum*** treatment (0.065)g with significant different compared with control treatment (0.326) g , followed by yeast extract agar 3 g /L treatment which gave (0.090) g and yeast extract agar 2 g /L treatment which gave (0.103) g followed by ***T.harzianum*** extract treatment (0.143) g and Bacterial factor treatment with (0.165) g with significant difference compared with control treatment which gave fresh and dry weight (3.267 , 0.326) g respectively. Material from living

ISSN 2072-3875

organisms include different polysaccharides and chitins or glucans and glycoproteins from microbial extract which added to the MS medium cause variety stresses effected decrease in fresh and dry weights(Siddiqui *et al* .,2010). Numerous studies on microbe elicitors interaction have identified microbial molecules that act as elicitors to increase the biosynthesis of plant secondary metabolites as a responsible for increase and interactions of their component which added to the medium (Angelova *et al* ; 2006).

Table (2) :Effect of biotic factors on callus fresh and dry weights /g

Treatment	Callus fresh weight /g	Callus dry weight/g
control	**3.276**	**0.326**
Bacteria	**1.642**	**0.165**
***T. harzianum* extract**	**1.467**	**0.143**
T. harzianum	**0.615**	**0.065**
Yeast extract agar 1g/l	**1.113**	**0.111**
Yeast extract agar 2g/l	**1.017**	**0.103**
Yeast extract agar 3g/l	**0.949**	**0.090**
LSD	**0.168**	**0.028**



Fig(3): callus with different biotic factors . A: *T.harzianum* B: extract yeast **C:** *A .chroococcum* **D:** Control

Fungal factors altered expression of several transcript , including representative from the phenylpropanoid ,pentose phosphate , glycolytic , and fatty acid metabolic pathways ,suggest that the response to biotic factors are much more than the simple induction of biosynthetic enzymes of secondary

ISSN 2072-3875

metabolisms. ***Azotobacter* sp** store Nitrogen in the cells in protein formula for nutrition and after analysis these proteins sedimentation in the medium cause stresses, and cytological variations include papilla formation ,increase cytoplasmic streaming and nuclear migration which are connected with depolymerization of microtubules and microfilaments (Kombrink and Schmelzer , 2001). Yeast extract agar adding to the medium and Bacteria elicitors showed inhibition in fresh and dry weights but total secondary metabolites are were increased (Han *et al* ; 2005). yeast extract agar contains numerous vitamins and nutrient materials like nitrogen which essential for building proteins ,enzymes which controls all important bioactivities and nucleic acids for cells divisions(Tawfiq ,2010) .

Catalase activity determination :

The results in Table (2) showed that the abiotic factors have different effects on catalase enzyme activity, the highest activity was for *T. harzianum* treatment (61.49) unit, Bacterial treatment (60.60) unit with significant different compared with control treatment and Yeast extract agar 2g/l treatment (57.68) unit respectively with significant different compared with control treatment. Plant extraction treatment (37.89) unit with significant different compared with control treatment .While materials from Fungal and Bacterial extracts include different polysaccharides from cells and cell wall chitine ,glucan , glycoprotein , and organic nitrogen stimulate defense reactions of the plant cells (Siddiqui *et al* ,2010) .After elicitor recognition , sequences of cytological variation and biochemical reactions have been identified in plant cells include papilla formation , increase cytoplasmic streaming and nuclear migration in the H, K , Cl , Ions and Ca ion Fluxes across the plasma membrane and development of reactive oxygen, oxidative cross linking of cell wall ,proteins increase catalase activity as mechanism for defense against biotic stress (Namdeo ,2007).

Total soluble carbohydrates determination :

Results in Table (2) showed that all treatments increase total soluble carbohydrates with significant difference compared with control treatment (without any factor) (110 . 333) ppm . The highest value was for ***T. harzianum*** treatment (315 . 330) ppm and for yeast extract agar 3 g/l treatment (324. 240) ppm followed by yeast extract agar 2 g / L treatment (308 . 422) ppm. Plant extract treatment gave (122 . 329) ppm. These increases of total soluble carbohydrates as responsible and adaptation of stresses (Muslim and Baqir , 2011). Analysis of polysaccharides in stresses condition cause increase in soluble carbohydrates as well as effect of stresses conditions on enzymes to form polysaccharides from simple sugar (Xu and Huang ,2010). These results agree with Smesim,2012 refer to increase of total soluble

ISSN 2072-3875

carbohydrates with biotic stresses using ***Bacillus subtillis*** (194.273) ppm compared with control treatment (174 . 142) ppm with significant difference in ***C. roseus*** in vivo. Zhao *et al.*,(2005) referred to the increase in total soluble carbohydrates using different fungal elicitors

Proline determination:

Results in Table (3) showed increasing in proline content as responsive to a biotic factors .The highest value was for yeast extract agar treatment 3g / L (1.208) µmol/g d.w , yeast extract agar 2 g /L treatment (1.121) u mol / g d.w and Fungal treatments ***T. harzianum*** extract and ***T. harzianum*** treatments gave (0.928 , 0. 900) µmol / g d.w respectively with significant different compared with control treatment (without any factor) (0,357) µmol /g d.w. Handa *et al* ., (1986) reported positive role for proline accumulation in adaptation of cells to change external water potentials .Obaid *et al* ., (2012) reported that increase in proline as responsible to biotic stresses .

Table (3) : catalase /unit , total soluble carbohydrates/ *ppm* and proline /µmol/d.w

Treatments	Catalase/unit	Total soluble carbohydrates/*ppm*	Proline /µmol/g d.w
control	**36.97**	**110.333**	**0.357**
Bacteria	**60.60**	**275.225**	**0.824**
***T. harzianum* extract**	**57.36**	**285.672**	**0.900**
T. harzianum	**61.49**	**315.330**	**0.928**
Y.E.A 1g/l	**40.61**	**140.337**	**0.705**
Y.E.A 2g/l	**57.68**	**308.422**	**1.121**
Y.E.A 3g/l	**48.36**	**324.444**	**1.208**
Plant extraction	**37.89**	**122.329**	**0.371**
LSD	**0.681**	**0.287**	**0.051**

The possible role of proline in membrane protection under stresses conditions .Proline increase under stress condition by deficit or analysis of protein and increase stability of plasmic membrane . Increase of protein considered protect adaptation by deficit of protiolytic activity act as regulator for different enzymes involved in protein formation. Proline act as save resource or relief for amino acids and proteins(Khatkar and Kuhad , 2000). Proline can protect plants from stress through different mechanisms, including osmotic adjustment, detoxification of ROS, protection of membrane integrity, and stabilization of proteins/enzymes (67).

Effect of biotic factors on vinblastine and vincristine alkaloids product from callus of *C. roseus* by (HPLC):

ISSN 2072-3875

The results in this study showed that all the treatments of biotic cause increase in the quantities of vinblastine and vincristine alkaloids in callus tissue. Quantities of vinblastine and vincristine alkaloids in all treatments determinate using HPLC . Absorbance peak of standard of vinblastine and vincristine sample with retention time(20. 72 ,12.988) Fig(4) which closed to retention time to retention time of vinblastine and vincristine in crude leaves extract of parent plant.

Effect of Yeast Extract Agar :

Results in table(3) showed that additions of biotic factors increase the quantities of vinblastine and vincristine in all treatments compared with control treatment with significant different. The highest quantity was for biotic factor yeast extract agar 2 g/L treatment (69.56 , 35.75) µg/g for vinblastine and vincristine respectively with significant different compared with control treatment (1.63 ,1.75) µg/g (d.w) with (42.67 ,20.42) folds for vinblastine and vincristine production respectively .(yeast extract agar 1g /L treatment (67.10 , 30.20) µg /g (d.w) with (41.16 , 17.25) folds of vinblastine, vincristine production respectively .Followed by Yeast extract agar 3g/l treatment (47.86,20.82)µg/g (d.w) with (29.36,11.89) for vinblastine and vincristine production respectively .Yeast extract agar treatments increase vinblastine and vincristine with the highest results and these results are similar to those obtained by Al-Mufarriji ,(2010) and Coery *et al*., (2005) who indicated that yeast extract agar increase metabolites levels at moderate and high concentration .Al-Abady, 2010 reported that addition of yeast extract to the medium of date palm callus inducing callus growth . Yeast extract contains IAA and GA3 and amino acids and proteins which induce the growth and division of cells but in high concentration cause stresses and accumulation of alkaloids for defends .(Tawfiq., 2010) .

Effect of *T. harzianum* treatment :

T. harzianum treatment was the third treatment gave (59.76 , 34.90) µg/g (d.w) for vinblastine and vincristine respectively with significant different compared with control treatment with (36.66 , 19.94) folds for vinblastine and vincristine production respectively . An improved catharanthine synthesis in ***C. roseus*** cell culture was observed by combination of fungal preparation enhanced the alkaloids accumulation (Zhao *et al* ., 2001) . Meenakshikoul *et al* (2003) indicated the production of valuable metabolites from *C. roseus* and from rich microflora was residing inside the plant tissues. Zhang *et al* (2003) isolated vincristine from ***Fusarium oxysporium*** .The relation between endophytic fungi and their host plant ,some available strategies for efficiently promoting production of these bioactive compounds and Some endophytes have the ability to produce the same or similar bioactive compounds as those originated

ISSN 2072-3875

from their host plants (Zaho *et al* .,2010).

Effect of *T. harzianum* extract treatment :

T.harzianum extract treatment gave (51.03 , 31.12) µg/g (d.w) for vinblastine and vincristine respectively with significant different compared with control treatment with (31.30 , 17.78) folds for vinblastine and vincristine production respectively. Ashutush *et al*. (2013) reported that different concentrations of fungal extracts of ***Aspergillus niger ,Penicillium notatum*** and yeast extract and chitosan enhance the synthesis of psoralen in ***Psoralea carylifolia*** suspension cultures . Siddiqui *et al* .(2010)summarized the role of elicitor by the following essential events in secondary metabolites (Binding elicitor to plasma membrane receptor, changes in Ca ions flux from and to cytoplasm ,decrease of pH of cytoplasm and activation of NADPH oxidases and protein phosphorylation patterns and protein kinase activation , change in cell wall structure (lignification) and in generating oxygen species, Synthesis of JA and SA as secondary messengers , and activation of genes that produce defends molecules like phytoalexins and other secondary compounds including alkaloids.

Table(3) Effect of biotic factors on vinblastine and vincristine alkaloids product from callus of *C. roseus* by (HPLC)

Biotic treatments	Vincristine µg/g (d.w)	Vinblastine µg/g (d.w)
control	1.7500	1.6300
Bacteria	31.0000	37.7000
T. harzianum Extract	31.1200	51.0300
T. harzianum	34.9000	59.7600
Yeast extract 1g/l	30.2000	67.10
Yeast extract 2g/l	35.7500	69.56
Yeast extract 3g/l	20.8200	47.8600
Plant extract	3.5500	2.3600
LSD	0.163	0.155

ISSN 2072-3875

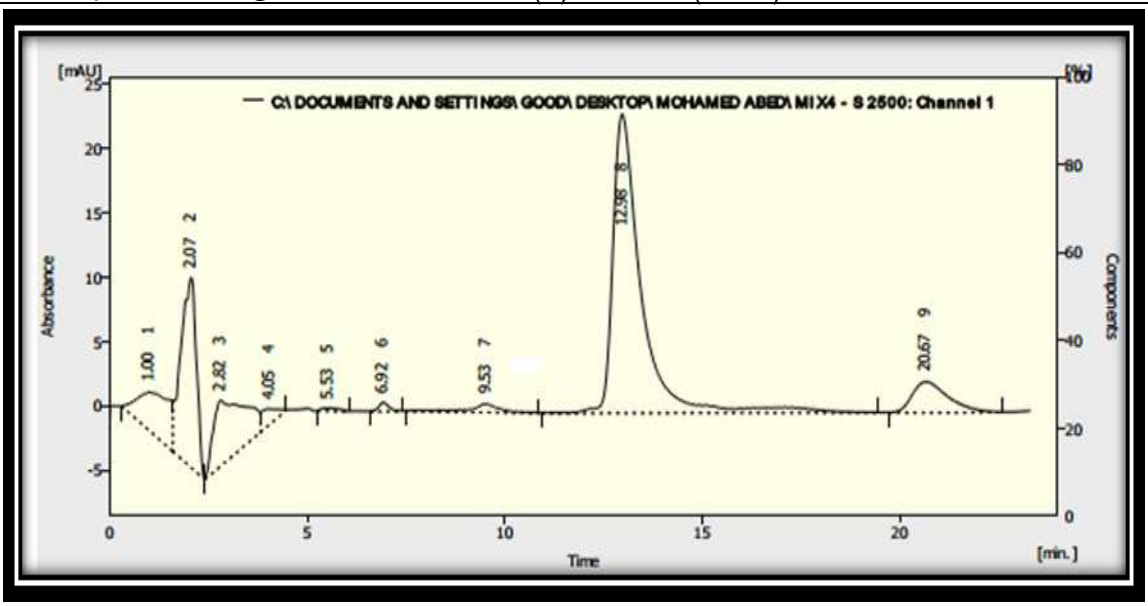


Fig (4) Peak area for standard curve of vinblastine and vincristine by (HPLC)

Eufrocinio .(2002) reported that the dual culture of ***T. harzianum*** and *C. roseus* produce an antimicrobial compound with remarkable activity against the Gram positive bacteria ***Staphylococcus aureus*** and ***Bacillus subtillis*** and named it Trichosetin. In ***C. roseus*** cell suspension culture Zhao et al .(2001) employed biotic elicitors derived from 12 fungi in order to test their effect on indole alkaloid production .They reported that different indole alkaloids were stimulated by different fungal mycelium. Proline and total soluble sugar content was higher in callus tissue than leaves of mother plant have showed positive correlation with vinblastine and vincristine production in callus tissues compare parent plants species as shown in (Table 3). Conclusively, the level of biotic treatments in callus medium showed considerable effect on total carbohydrate and vinblastine and vincristine production accumulations.

References :

Ajay N. (2002).Influence of Fungal Elicitors on Production of Ajmalicine by cell cultures of C. roseus. India Biotechnol .prog . 18: pp 159-162.

Al-Abady,U.A.M. (2010). Study the effect of yeast extraction and 2ip on embryogenesis callus growth of date palm p. dactylifera L.CV. Ashkar.Dep. of biology college of sciences Misanuniversity :51 (4) : 607-615.

AL-Hatamy ,K. T. (2006). Comparative studding between *Daturainnoxia*Mill and *Datura metal* Linn with respect to production of Tropan alkaloids *in vivo* and *in vitro*.phd in Biological Botany ,Sciences college ,Babylon university.

Al-Hattab ,Z.N.;Al-Kateeb ., E.Al-Quadhy , W.K. and Mahdi , G.

ISSN 2072-3875

(2000).Effect of growth hormons on tropane alkaloids production in *Datura Metal* callus cultures . College of pharmacy ,Baghdaduniversity.Vol .13 (1)PP .

Al- Mafarji , K.E.R.(2010). Stimulating production essential oils and Rosmarinic acid from *RosmarinusOfficinales*using Biotic and abiotic Effects.Vol. ( )

Angelova Z., Georgiev, S .; Roos W.(2006). Elicitation of plants . Biotech. Leu 33(2) : 381-385.

Ashutosh, K. ;Deepak, p and Absar A.(2013).Isolation , purification and characterization of vinblastine and vincristine from indophytic fungus *Fusariumoxy sporum* isolated from *Catharanthus roseus .Dol:10.137rJournal.pone.0071805*

Bates,LS., Waldren . RP .andTeare ,JD. (1973) . Rapid determination of proline for water stress studies . Plant soil , 39 : 205-207.

Carew , D.P. and Krueger , R.J.(1977). *Catharanthus roseus* tissues culture :The effect of medium modification on growth and alkaloid production. Planta Med.40 (4) :326-336.

Corey ,D.B., David ,V.H. Mohammed , A.F., Joel, T., pedro,M.R. and Lioyd ,W.S. (2005). Metabolic profiling of *Medicagotruncatula*cell .culture reveals the effects of biotic and abiotic elicitors on metabolism.J.ofExp . Bot., 56(410) : 323-336.

Cragg, G.M. and Newman, D.J. (2005). Biodiversity : A continuing of novel Drug Leads . Pure appl. Chem. 77(1): 7-24.

Davies,P.J. (2004). Plant Hormones. Biosynthesis , Signal Transduction , Action. Kluwer Academic Publishers.

Ethbaieb ,E. G.(2010). Using of tissues culture for vinblastine and vincristine production in *C . roseus in vitro* , a college Agriculture, University of Kufa.

Eufrocinio,E.,Marfori,Shinichiro,Kajtyyama, and Ej-jchirofu Kusaki. (2002).Trichosetin , a novel tetramic acid ,Antibiotic produced in dual culture of *T. harzianum*and *C. roseus*callus .Biotech.Graduate school of engineering.Osaka university ,Jaban ,57C, 465- 470.

Fumihiko,S.; Hashimoto ,T.; Hachiya , A.; Tamara ,K .; Choi ,K. ; Morishing , T.; Fujimoto , H. and Yamada , Y . (2000). Metabolic engineering of plant alkaloid biosynthesis .Academy Sci ., (3): 367-372.

George , E. F. ; Hall and G.J. De Klerk .(2008). Plant propagation by tissues culture. The Background 3rd Edition, Published by Springer ,Dordercht. The Netherlands.

Han YM, Oh H, Na M, Kim BS, Oh WK, Kim BY, Jeong DG, Ryu SE, Sok DE & Ahn JS.(2005). PTP1B inhibitory effect of abietane diterpenes isolated from *Salvia miltiorrhiza*. Biol Pharm Bull. 28: pp. 1795–1797.

Handa, S. ,Handa AK , Hasegawa P.M and Bressan RA.(1986).Proline

ISSN 2072-3875

accumulation and the adaptation of cultured plant cells to water stress. Plant physiol., 80 :936-945.

Harborne, J. B. (1984).Phytochemical Methods: A Guide to modern Techniques of Plant Analysis, 2nd ed. Chapman and Hall, London.

Khatkar,D and Kuhad, S .(2000). Short term salinity induced changes in two wheat cultivers at different growth stages .Biol . Plant 43 (4) :629-632.

Kombrink E, Schmelzer E.(2001) . The hypersensitive response and its role in local and systemic disease resistance . European J plant Path 107 :69-78.

Kumar R.; Bhatia, B ; Kukreja, R.; Behl R..; Dudejas, K and Narulan. S (2007). Establishment of *Azotobacter* on plant roots chemotactic response, development and analysis of root exudates of cotton *(Gossypiumhirsutum* L.) and wheat (*Triticumsativum*).Journal of Basic Microbiology 47(5): 436-439.

Levesque , R.(2007). SPSS programming and Data Management : A guide for SPSS and SAS users (4 thed).Chicago lllinois Lnc ISBN 1-56827-390-8.

Meenakshi k.,Nehas , L.;Akra ,Ramish , C. and Sheela,C .(2013). *Catharanthusroseus*and prospects of its Endophytes:Anew Avenue for production of bioactive metabolites.International Journal of Pharmaceutical sciences and research.Vol 4 (7) : 2705-2716.

Muslim ,A.A. and Baqir ,S.M. (2011). Effects of water stress on the leaves of peroxidase and catalases enzymes , proteins and carbohydrates in grape callus of *Amber queen* and *Cardina*l CVs. *In vitro* . Journal of Kuffa for Agriculture sciences .(3): 143-150.

Murashige, T. and Skoog, F. (1962). *A revised medium for rapid growth and bioassays with tobacco tissue cultures*. Physol. Plant. 15: 473-497.

Namdeo ,A.G. (2007). Plant cell elicitation for production of secondary metabolites: A review .PharmacognosyRev ., 1(2-3) : 69-79.

Obaid,H. ;Esam ,F. and Yanas ,L. (2010). Morphological and physiological responses to *Maluscommunis*and *Malustrilobata*,for water stress.Department of horticulture science faculty of agriculture research Duma, Damascus Syria.28 (2): pp 143-159.

Ozden M, Demirel U, Kahraman A. (2009). Effects of Proline on Antioxidant System in Leaves of Grapevine (Vitis vinifera L.) Exposed to Oxidative Stress by H2O2. Scientia Horticulturae119, 163-168.

Siddique Z.H.; Mujib ,A.;Ahmed M.M and Ali, A .(2010). Fungal elicitors : A potent approach for enhancing secondary metabolites in cultured cells. In Gupta VK, Tuohy M, Gaur RK (eds) Fungal biochemistry and

ISSN 2072-3875

biotechnology . Lap Lamber Academic publishing AG and CO. KG, Germany, pp : 88- 104.

Semysim ,E .(2012). Effect of BBacillusKB1 ,Paclobutrazol and KT-30 on growth *C. roseus*and production of vinblastine and vincristine alkaloids college of sciences ,university of Kuffa.

Taha , H.S. ; El-Bahr , M.K. and Seif – Nasr ,M. M.(2008). In vitro study on *C. roseus*.Manipulation of some amino acid as precursors for enhanced of indole alkaloids production in suspension pp 123-153.

Tawfiq , A.A. ,(2010). Estimation leaves of indol acetic acid (IAA) and Gibberellic acid (GA3) from dry bakery yeast *Saccharomyces cereviciae*.Biotechnology research center ,Baghdad university ,college of science for women ,4 (2) pp 412-423.

Xu,C. and Huang B. (2010). Differential proteomic responses to water stress induced by PEG in two creeping bentgrass cultivars differing in stress tolerance .J. plant physiol .,(v)167 :1477-1485 .

Zhang C.,Jain Y.(2003).Ethylene inhibitors enhance elicitors-induced paclitaxel production in suspension culture of *Taxus*spp cells .Enzyme Microb Technol. 32: 71-77.

Zhao J, Davis LC and Verpoorte R.(2005). Elicitor signal transduction leading to production of plant secondary metabolites. BiotechnolAdv23 : 283-333.

Zhao J., HuQ.,Guo Y.Q and Zhu W.H.(2001). Elicitor induced indole alkaloid biosynthesis in*Catharanthusroseus* cell cultures is related to Ca ions influx and the oxidative burst. Plants sciences vol (61):423-431.

Zhao,J. and Verpoort , R. (2007). Manipulating indole alkaloid production by *C. roseus* cell culture in bioreactors: from biochemical processing to metabolic engineering .Phytochem .Rev. , vol(6): 435-457.

Zhao,J. ;L. Zaho,J.; Wang ,T.S ,L. Zhong.; X. L , and Gao ,X. (2010). Indophytic Fungal for producing bioactive compounds originally from their host plant.China Agricultural University, (v)160 :147-150

ISSN 2072-3875