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Comparative Study between using of Biomass and Extract of Trichoderma Viride to Inducing Anticancer (TIA) Vincristine Production from Catharanthus Roseus in Vitro

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Abstract
This experiment was conducted in college of Agriculture , Al-Qasem Green university in 2015 ,to study the different of effects between using *Trichoderma viride* as biomass and as extract to induce the production of anticancer vincristine in vitro using MS medium supplemente with (2,4-D) and (BA) in (1+1) mg /L concentration to induce callus production then inoculated the callus directly with biomass of *T.viride* by single spore .In first method and adding extract of *T.viride* to the MS medium in the second method .The study include quantity and quality determination of vincristine by HPLC technique .Results revealed that the highest values was for the using of *T.viride* as biomass which gave(25.120)µg/g vincristine and the lowest value was for *T.viride* as extract which gave(15.346)µg/g vincristine .also results explained that adding of *T.viride* as biomass and the extract cause decrease in the fresh and dry weight of callus inducing from leaves of *C.roseus* and the highest decrease was to biomass method (200, 0.019) mg followed by extractmethod(340,0.031)mgrespectively.
Keywords: *Trichoderma viride in vitro, vincristine* extract.

Introduction

Medicinal plants considered very important origin to study for humankind(Cragg and Newman,2005).The products of plant have many applications include(drugs,pharmaceuticals ,dyes) and active and important (TIA)compounds include vincristine as a good anticancer (Zhao *et al.*,2005). *Cantharanthus roseus* (fig,1)considered one of the important plant to product many active compounds such as anticancer vincristine (AL-Hatamy,2006).This compound was produce in plant refer to as secondary metabolites to protect the plants against microorganism and animals and enhance the competitive with other plants. Vincristnie was product in aerial part in leaves of *C.reseous* but in very few quantity and affected by environmental condition (faccchini,2001) also affected by external biotic factors such as fungi (zhao *et al*,2007). Ajmalicine accumulation increased by 3-folds when using biomass of *Aspergillus niger* ,*Fusarium moniliform* , T.viride (Ajey Namdeo 2002) .This study aims to increase the production of anticancer the production of anticancer vincristine by two methods using *T.viride* and comparative study between these methods.

Fig-1 :*Catharanthus roseus*

2-Material and methods

2-1 culture medium preparative: MS medium was used for tissue culture in the form of packets with weight of 4.4 grams ,used for preparation of 1L of MS medium supplemented with (1+1)mg /L (2,4-D)and (BA). Sucrose was added at 30g/L and agar at 8 g/L, PH controlled on 5.8 then transferred to autoclave for sterilization on 121 c and 1.04 kg/cm^2 for 20 minutes and left until cool then placed in screw cup tubes (2,5x10) cm (Anjelova *et al*,2006).

2-2 leaves tissue culture:-
the leaves of *C.roseus* were collected and washed by tap water several times and transferred to laminar air flow cabinet for surface sterilization by 70% ethanol for 30 seconds and washing by distilled waterseveral times and

by 6% for 20 minutes sodium hypochlorite then washed 3 times by distilled water then placed pieces of the leaves in │1x1 │cm on the MS medium in each of screw cup tube then transferred to the growth chamber in suitable condition 8/16 hr photoperiod and 25 ċ (Al-Hatamy ,2006).

2-3 *T. viride* preparation inoculums

2-3-1 : first method ,by single spore method .

After callus harvesting and sub culturing on MS medium after 45 days from first culture 250 mg of callus was inoculated by single spore of *T.viride* in the glass tube directly ,closed well, transformed to growth chamber at 25 ċ and 16/8hr photoperiod till the fungus covered the callus (Eufrocinio *et al*.,2002)

2-3-2 second method

Using fungal extract by cork borrer 3 disks were taken from exit edge of pure growth of *T.viride* perti-dish and placed in flask with 250 ml of PD broth closed well, transferred to inocubators for 7 days, then the fungal extract was taken by Buokhner funnel with filtration paper whattman No.1 and vacuum pump, (0.1)ml from fungal extract v/v was added to the MS medium with all the steps of preparation of MS and sterilization supplemented with growth regulators (1+1)mg/L 2,4-D and BA in screw cup tubes ,then transferred to growth chamber at 25 c and 16/8 photoperiod after placed 250 mg of callus on the surface of the MS medium(Cragg and Newman,2005).

2-4 Preparation of callus tissue for alkaloids extraction.

Alkaloid compounds were extracted from callus tissues ,inducing phase, at the best combination according to AL-Hattab *et al* (2000) as the following:

1- Aquantity of 500 mg from dry callus was crushed well in mortar casserole, after drying fresh callus in oven on 40 C for 24 hr.
2- The dry callus was mixed with ethanol 80% and ether 20 % at 1:4(v/v) ratio.
3- Ammonium hydroxide 25% was added, putting on shaker for 3 hr.
4- Hydrochloric acid 5% was added .
5- The extraction was mixed with acidic ethanol 1 : 3 (v/v).
6- Mixture was filtrated by whattman No 1 paper.
7- Ammonium hydroxide was added to make high pH up to 9.
8- The basic solution was extracted with chloroform thrice and the down layer was taken each time.
9- Anhydrous sodium sulfates were added and left to dry on 40 C for 24 hr.

2-5 Quantity and quality determination of vincristine compound in callus extraction by (HPLC) technique.

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

2-6 Caliberation curves

One mg from pure vincistine was dissolved in 9 ml from HPLC grade water to obtain 100 mg/ml and by serial dilutions to obtain on 10 mg /ml ,the concentration of vincristine in callus was estimated by :-

Vincristine quantity = $\frac{\text{Relative area for sample}}{\text{standered Relative for standared solution}}$ x Alkaloid concentration in standared sample x dilution factor

Statistical analysis:

statistical analysis was according to complete random design, ten replicates were used for each treatment and were tested by the least significant different (L.S.d) at probability level 5% according to system of SPSS (Leavesquare,2007).

3- Results and discussion

3-1 effect of *T. viride* on fresh and dry weight using biomass inoculation and extract adding treatments.

Table 1: Effect of *T. viride* on callus fresh and dry weight

Treatment	Callus F.W mg	Callus D.W mg
Control (without Fungi)	350	0.035
Callus (with extract of Fungi)	340	0.031
Callus (with Fungi biomass)	200	0.019
L.S.D	79	0.008

***Each number was average of 10 replicates.**

The result shown in table (1) and (Fig 2 , 3 ,4)referred that the inoculation of Callus by biomass of *T. viride* gave the lowest values in fresh and dry weight which equal to (200 ,0.019) mg respectively but the treatment of

Callus by adding the extract of *T. viride* to the MS medium in 0.1 v/v gave the highest values (340,0.031) mg with significant different compared with control treatment .

Fig(2): control treatment (Callus without fungi)

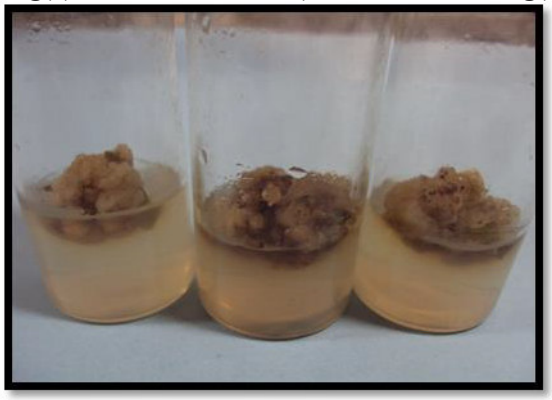


Fig (3): Treatments of T. viride extract with 0.1 v/v with MS medium of tissue culture.

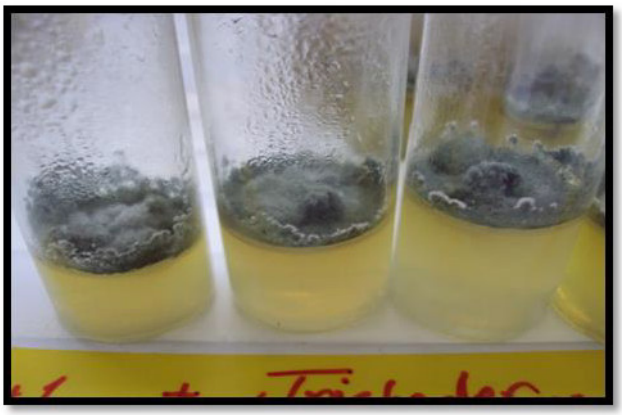


Figure 4:callus with fungi as biomass

Table 2: Effect of *T. viride* on vincistine product from Callus of *C.roseus* in vitro.

Treatment	Vincristine µg /g
Control	2.780
T. viride as biomass inoculation directly	25.120
extract *T. viride* as adding to MS medium	15.346
LSD	0.820

***Each number was average for 10 replicates**

The result table (2)and Fig (5,6,7,) explained that the adding of *T. viride* as biomass to the Callus cause increase in anticancer vincristine with highest value (250.120) ug/g followed by treatment of adding *T. viride* as extract to the MS medium cause increase in quantity of vincistine with the lowest value (15.346) with significant

different compared with control treatment (Callus without Fungi which gave (2.780) ug/g vincristine .

The materials from living organisms include different substances such as poly saccharides and chitins or glycoproteins from microbial extract and different materials from biomass which caused different stresses in the cells of plant and callus caused decreased in fresh and dry weight of Callus and increase in active compounds to use it for defend against the unsuitable conditions around the cells of plant (siddique., 2010). Many studies on microbial elicitors such as fungi explain that the adding of fungi contains many different microbial molecules that act as elicitors to increase the biosynthesis of plant secondary compounds as responsible for this adding and interaction with cell and cell wall of plant cell and cell wall of the callus cells cause exchanging in the intery and exit of the different elements from and to cells cause accumulation of these active compounds against these exchanginds (Angelova etal ,.2006).

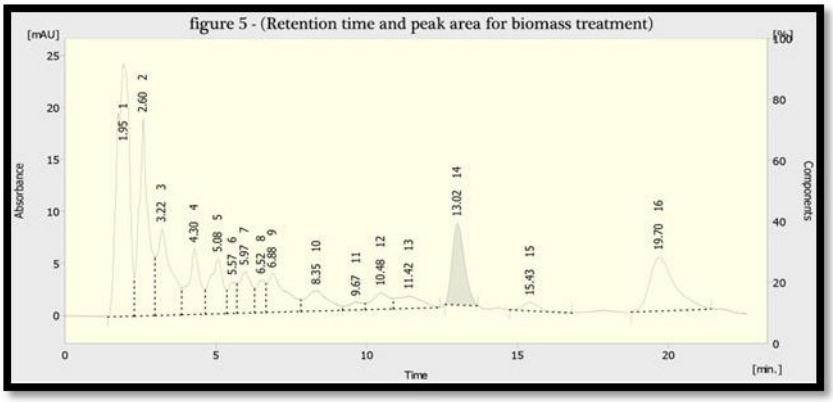


Fig 5 :Retention time and peak area for biomass treatment

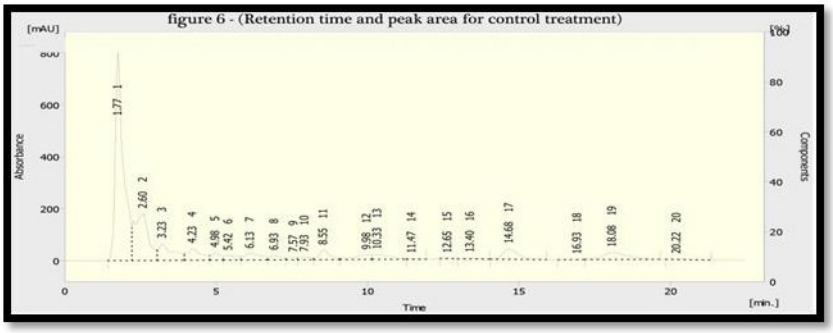
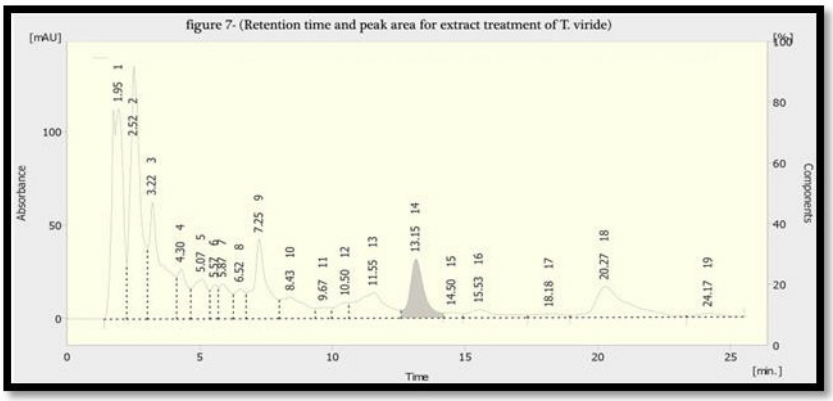


Fig 6 : Retention time and peak area for control treatment



Retention time and peak area for extract treatment of T. viride Fig 7 :

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