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IMPACT OF DIFFERENT PLOIDY LEVELS CHANGE ON DIANTHALEXIN CONTENT IN *DIANTHUS CARYOPHYLLUS* L.

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Abstract – The research was conducted to study the effect of elicitor (*Fusarium oxysporum*) and precursor (anthranilic acid) on accumulation of dianthalexin in callus culture initiated from leaf of *Dianthus caryophyllus*. Callus was induced in Murashige and Skoog (MS) media in the presence of 1.0 mg/L Dichlorophenoxy acetic acid (2,4-D) and 0.5 mg/L Benzyl Adenine (BA), supplemented with *F. oxysporum* (0.0, 2.0,4.0 or 6.0) mL/L and anthranilic acid (0.0, 1.0, 5.0 or 10.0) mg/L. Quantitative and qualitative assessment of dianthalexin was performed by using high performance liquid chromatography (HPLC) after three and six days culture. Cytological study was performed on callus treated with *F. oxysporum* and anthranilic acid by using Paradichlorobenzen (PDB) for chromosomes pretreatment and dimeric cyanine nucleic acid stain (YOYO-1) for staining. Anthranilic acid at 10.0 mg/L was the most effective for stimulated dianthalexin in callus after three days gave 102.46 µg/mL followed by *F. oxysporum* at concentration 4.0 mL/L after three days too, gave 73.74 µg/ml comparison with dianthalexin in leaf of intact plant 6.33 µg/mL and in untreated callus 28.15 µg/mL. Addition of *F. oxysporum* and anthranilic acid lead to change in chromosome numbers. Dianthalexin accumulation in treated callus coupled with chromosomal variation especially in callus treated with 4.0 mL/L *F. oxysporum* and 10.0 mg/l anthranilic acid which have tetraploid cells.

INTRODUCTION

Plant produces a wide array of products which have utility for human health. A lot of plant compounds are used to treat infection or fighting diseases such as stroke, cancer and heart problems. The knowledge increased in plant secondary metabolites, coupled with low produce stimulated research on possibility for increasing the level of metabolites in plants, therefore number of options are available, especially in plant biotechnological field (Caruso *et al.,* 2013). Through *in vitro* culture, secondary metabolites able to produce all year around and allow to use elicitors that enhance or induce metabolites production. Ajmalicine product in *Cantharanthus roseus*, was increased after useing *Fusarium* and *Aspergillus* as elicitor (Rodrigues *et al.,* 2014). Metabolic engineering can improve production of secondary metabolites by modifying the pathway of interest, based on overexpression of significant genes silencing (Taha, 2016). In the last few years, addition options based on scaling up the ploidy level metabolite-producing plant have attracted the attention of scientists. Polyploidy used in several crop such as potato, banana, apple and sugar beet. In medicinal plants polyploidy are usually more valuable because they exhibit increased content of bioactive compounds, for example in *Papaver somniferum* used polyploidy as a valuable tool to produce high morphin (Caruso, 2013). The aim of this paper is find the change in ploidy level has affect on dianthalexin amount in *Dianthus caryophyllus* after treated with elicitor and precursor.

MATERIALS AND METHODS

Tissue culture

The best combination for callus induction was

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chosen based on Alwash *et al.,* (2017), by using MS medium supplemented with 1.0 mg/L 2,4-D and 0.5 mg/L BA for inducing callus from leaf explants of *D. caryophyllus*.

Preparation of *F. oxysporum*

For preparation *F. oxysporum* followed the method described by (Rashid *et al.,* 2011) and added to culture media in different concentrations (0.0, 2.0, 4.0 or 6.0) mL/L.

Preparation of anthranilic acid

Culture medium supplemented with precursor; anthranilic acid in concentrations (0.0, 1.0, 5.0 or 10.0) mg/L.

Extraction and estimation of dianthalexin

Dianthalxin was extracted from intact plant, treated and untreated callus by taking 2 g of leaves (intact plant) and callus were dried at room temperature, grinded into powder separately. Crude extract was obtained by mixing the powder with 20 mL (70%) of methanol for 6 hours in ordinary reflex at 60- 80 ºC, materials filtered after cooling. Filtrate was evaporated at room temperature and the extract stored in a refrigerator at 4 °C for future use. Dianthalexin was estimated qualitatively and quantitatively in crude extract by using HPLC analysis. The main compounds were separated on fast liquid chromatography (FLC) column beneath the optional conditions:

Column: phenomenex C-18, 3µm size of particle (50×2.0mm I.D).

- Mobile phase: 0.5% phosphoric acid: methanol, 23:70, v:v
- Detection: under UV at 275 nm.
- Injection volume: 20µL
- Flow rate: 1.4 mL/min.

The concentrations were calculating as follow (6):

Concentration of compound (µg/g)= $\frac{peak\ area\ of\ compound}{peak\ area\ of\ standard}$ × concentration of standard× dilution factor.

Cytological analysis

The roots (intact plant) and callus segments pretreated with saturated solution of paradichlorobenzen (PDB) for 4 hours at 16 °C, then dropped in Carney's fixative solution (3:1) ethanol: acetic acid for 12 hours. Fixative tissues staining were done with YOYO-1 according to (Schwarzcher, 2016).

RESULTS AND DISCUSSION

Table (1) showed that relation in level of anthranilic acid with dianthalexin accumulation.

Dianthalexin was increased when used anthranilic acid at 10.0 mg/L after three days to (102.46) µg/mL (Fig. 1), while increasing the incubation period to six days reduced the accumulation of dianthalexin to (86.7) µg/mL. Followed by 5.0 mg/L with significantly decrease in dianthalexin to (57.6) µg/mL after three days, then after six days gave (36.62) µg/mL. All the phytoalexins detectable in infected carnations are derived from anthranilic acid. Anthranilate synthase catalyses the synthesis of anthranilic acid from chorismic acid, which is considered to be a central

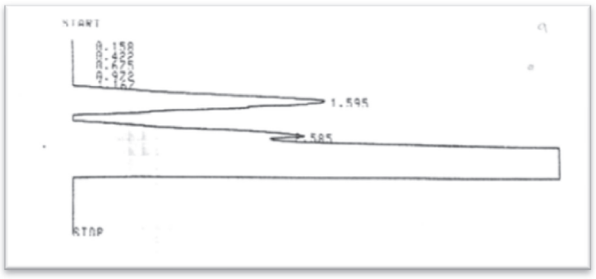

Fig. 1. HPLC analysis of dianthalexin in extract of callus treated with 10.0 mg/L anthranilic acid after three days culture.

Table 1. Effect of Anthranilic acid and *F.oxysporum* on dianthalexin quantity (µg/mL) estimation of *D. caryophyllus* callus after three and six days culture

	3 days				6 days			
Untreated-callus	Anthranilic acid		*F. oxysporum*		Anthranilic acid		*F. oxysporum*	
	Con. (mg/L)	HPLC (µg/mL)	Con. (mg/L)	HPLC (µg/mL)	Con. (mg/L)	HPLC (µg/mL)	Con. (mg/L)	HPLC (µg/mL)
28.15	1.0	20.3	58.19	2.0	1.0	8.9	1.0	38.70
	5.0	57.6	73.74	4.0	5.0	36.62	5.0	32.51
	10.0	102.46	68.05	6.0	10.0	86.7	10.0	25.73

precursor for dianthalexin (Matern, 1994).

Table (1)showed that treating callus with *F. oxysporum* extract led to an increased concentration of dianthalexin compared with untreated callus (Fig. 2). Treatment with 4.0 mL/L of *F. oxysporum* gave the best result 73.74 µg/mL dianthalexin after three days incubation (Fig. 3), but the yield of dianthalexin decreased after six days to 32.51 µg/mL, followed by callus treated with 6.0 mL/L of the same elicitor produced 68.05 µg/mL of dianthalexin after three days of culture, reducing to 25.73µg/mL after six days of incubation. This compares with 28.15 µg/mL of dianthalexin extracted from untreated callus and 6.33µg/mL extracted from leaves of intact plant (Fig. 4). This result is in agreement with (Petro *et al.,* 2013), who reported similar results for stimulating Dianthalexin in culture of *Dianthus caryophyllus* treated with fungal elicitors.

Fig. 2. HPLCanalysis of dianthalexin in *D. caryophyllus* untreated callus extract

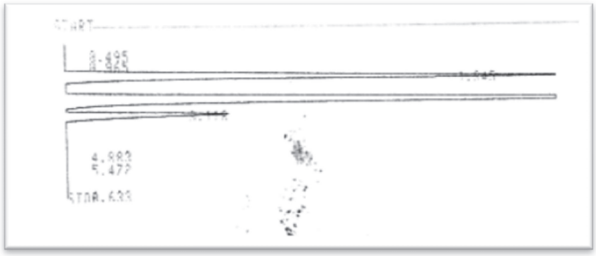

Fig. 3. HPLC analysis of dianthalexin in extract of callus treated with 4.0 ml/L *F. oxysorum* after three days culture.

Fig. 4. HPLC analysis of dianthalexin in *D. caryophyllus* leaf extract (intact plant)

Fig. 5. HPLC analysis of dianthalexin standard

Paradichlorobenzen (PDB) was more effective when used for pretreatment (Fig. 6) because chromosomes were more visible and more easily countable under the microscope. These results contrast with (Yaccob *et al.,* 2013), who pretreated root tips of *D. caryophyllus* in 0.002 M 8-hydroxyquinoline for 3 h at room temperature.

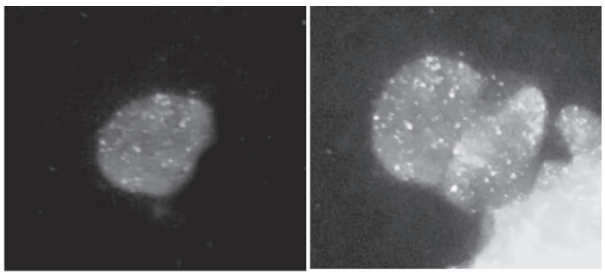

Fig. 6. Effect of Pradichlorobenzen on arresting of *D. caryophyllus* chromosomes of
A: root (intact plant) **B**: callus

PDB not only causes spindle inhibition but also lead to clarification of chromosomal constrictions due to contraction (Ghonema and Ibrahim, 2010).

In present study, we observed a significant increase in dianthalexin level after addition *F. oxysporum* and anthranilic acid indicated rapid response of callus cell, as well as the ability of additives to stimulate dianthalexin biosynthesis. Callus treated with *F. oxysporum* and anthranilic acid in different concentrations, show significant differences in chromosomes number compared with root and untreated callus, but the majority present 2n = 30 chromosomes in all treatments (Table 2).

Treated callus with *F.*oxysporum (4.0mL/L) appeared cells (1%) with tetraploid after 3 days culture, increased culture time to 6 days led to increase in the percentage of tetraploid to 1.4%. Also tetraploid cell show when treated callus with

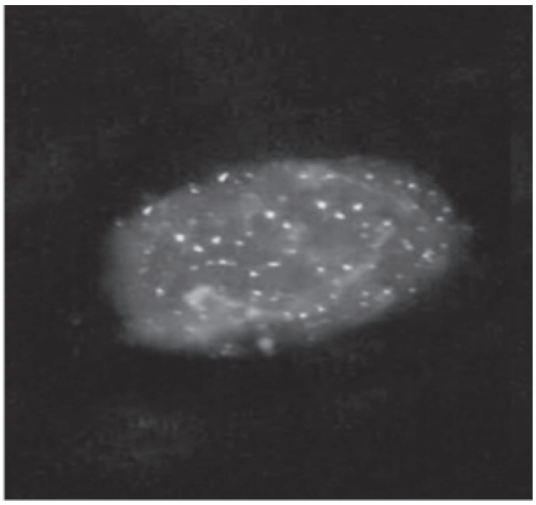

Fig. 7. Callus cell of *D. caryophyllus* with 60 chromosomes (tetraploid) when treated callus with anthranilic acid (10.0 mg/L) after six days.

anthranilic acid at 10.0 mg/L in percentage 2% at 3 days (Fig. 7) then reduced after 6 days culture to 1.5%.

Stress influence the ability of tissue to adjust to new conditions and initiate developmental transition for survival. These changes are facilitated by reprogramming cellular physiology, metabolism changes and revival of cell division (Petrov *et al.,* 2015).

Plants respond to biotic stress by upregulating the expression of a large number of pathogenesis-related (PR) genes, a process that seems to play a role in plant's defense mechanisms. Evidence from several studies has shown that PR genes play a role in physiological processes during plant growth and development, and that their expression is regulated by factors including stress and plant hormones. New genomic methodologies are increasingly being employed to identify the genes associated with stress. Using a variety of elicitors, these studies have revealed that, in addition to PR genes, hundreds of genes, other genes exhibit differential expression after the activation of a defense of program. Furthermore, patterns of protein accumulation change both qualitatively and quantitatively in stressed cell. Different stresses are implicated in the regulation of different but overlapping series of genes and complex networks of plant signalling pathways. Gene expression is controlled at different levels operating through changes in DNA methylation, histone modification and chromatin remodelling. Stress can alter the pattern of gene methylation, which is activated under stress conditions (Yaccob *et al.,* 2013).

These results agree with (Sudarshana *et al.,* 2015) who noticed that polyploidization in *Chamomilla recutita* resulted in higher flavanoid content and observed increased tropane alkaloids in tetraploid *Atropabelladonna* plants. The improvement in the secondary metabolites content associated with polyploidy is a desired aim in the biotechnological process in order to target the molecule content in medicinal species (Rodrigues *et al.,* 2014).

Table 2. Effect of different concentration of *F. oxysporum* and anthranilic acid, in chromosomes number of callus cell (%)

Treatment	Diploid (%) 2n= 30	Cells with more than 30 chromosomes (%)	Cell with less than 30 chromosome (%)	Teteraploid cell (%)
Intact plant (root)	100	0	0	0
In vitro (untreated callus)	100	0	0	0
Callus with (2.0 ml/L) F.oxysporium after 3 days incubation	38.0	30.2	31.7	0
Callus with (2.0 ml/L) F.oxysporium after 6 days incubation	34.1	33.2	32.7	0
Callus with (4.0ml/L) F.oxysporium after 3 days incubation	37.0	32.5	30.5	1
Callus with (4.0ml/L) F.oxysporium after 6 days incubation	34.6	32.3	31.7	1.4
Callus with (6.0ml/L) F.oxysporium after 3 days incubation	35.2	34.2	30.5	0
Callus with (6.0ml/L) F.oxysporium after 6 days incubation	34.6	32.9	32.4	0
Callus with (1.0 mg/L) anthranilic Acid after 3 days	34.8	30.6	34.6	0
Callus with (1.0 mg/L) anthranilic Acid after 6 days	35.1	30.2	34.6	0
Callus with (5.0 mg/L) anthranilic Acid after 3 days	33.7	31.8	34.4	0
Callus with (5.0 mg/L) anthranilic Acid after 6 days	33.8	31.5	34.6	0
Callus with (10.0 mg/L) anthranilic Acid after 3days	35.0	32.2	30.8	2.0
Callus with (10.0 mg/L) anthranilic Acid after 6 days	35.5	31.8	31.1	1.5

The increase in dianthalexin content as a result of somaclonal variation that is evidenced from our study could be due to the higher activity of enzymes that catalyzes its biosynthesis. The basic genetic material remains the same, but gene dosage is multiplied and there is potential to modulate gene expression and hence enzyme activity per unit protein (Belabbassi *et al*., 2017).

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