Research Article

5-azacytidine Increases Tanshinone Production in Endophytic Fungi of *Salvia abrotanoides* (Kar.) Sytsma

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Abstract

Endophytic fungi are often producing host plant metabolites. Tanshinones are secondary metabolites of the *Salvia* genus which are also produced by some endophytic fungi. Efficient secondary metabolite production in endophytic fungi drops significantly after sequential subcultures. 5-azacytidine (5-AC) is an analog of the naturally occurring pyrimidine nucleoside cytidine and a DNA methyltransferase inhibitor. In this relation, 5-AC is an effective tool to induce the expression of silenced secondary metabolite genes in fungi. We isolated 4 endophytic fungi from the roots of *Salvia abrotanoides* which produced tanshinone. Cryptotanshinone and tanshinone IIA were produced by *Penicillium canescens*, *Penicillium nodositatum*, and *Penicillium pinophilum*, while *Paraphoma radicina* only produced tanshinone IIA. The maximum amount of tanshinones was extracted from *P. pinophilum* culture with 130.826 mg cryptotanshinone /g of dry weight and 50.155 mg Tanshinone IIA/g of dry weight. These amounts were significantly more than tanshinones produced in plant roots (0.55 mg cryptotanshinone/g of dry weight, 1.3 mg Tanshinone IIA/g of dry weight). In the third subculture, tanshinone production decreased significantly. 5-azacytidine as an epigenetic modifier retrieved tanshinone production in the third subculture of *P. pinophilum*. Also, 5- azacytidine treatment made a big jump in Tanshinone IIA production in *P. radicina* (63.176 mg TIIA/g of dry weight) besides increasing Tanshinone IIA production in *P. nodositatum* cultures. This is the first report using 5- azacytidine to improve tanshinone production in endophytic fungi. Our results confirm that 5- azacytidine is an efficient, easy, and quick chemical to felicitate secondary metabolite production in endophytic fungi.

***Keywords*:** Endophytic fungi, Tanshinone, 5-Azacytidine, *Salvia abrotanoides*

Introduction

Salvia abrotanoides (Kar.), called "barazambel, belongs to the tribe stachyoideae-Nepeteae, Lamiaceae family. This species is distributed in wide regions of Asia, such as Afghanistan, the Himalayas, Turkestan, Pakistan, and Tibet (Rechinger, 1982). The roots of this medicinal plant are mainly used for the treatment of leishmaniasis in Iranian folk medicine (Jaafari et al., 2007). It also has a sedative, analgesic, antiseptic, and cooling effect (Hosseinzadeh and Amel, 2001; NASIRIASL et al., 2002; Moallem and Niapour, 2008). Some of the pharmacological effects of this plant such as antiplasmodial, anti-inflammatory, and cytotoxic effects have also been reported (Sairafianpour et al., 2001; Esmaeili et al., 2009; Beikmohammadi, 2012). Tanshinone is the most bioactive and frequent metabolite in *S. abrotanoides* (Sairafianpour et al., 2001). Roots of *S. abrotanoides* and *S. miltiorrhiza* are considered the most important resources of tanshinone IIA, affecting cardiovascular action.

In eukaryotic cells, gene expression is greatly influenced by the dynamic chromatin environment. Epigenetic mechanisms, including covalent modifications to DNA and histone tails and the accessibility of chromatin, creates various chromatin states (Chang et al., 2020). According to certain information, DNA methylation on the promoter and coding region of secondary metabolite (SM) genes induce heterochromatin formation and consequently

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gene silencing (Reyes-Dominguez et al., 2010). A multi-component methyltransferase complex is responsible for DNA methylation and heterochromatin formation in the fungi *Noreuspora crassa*, a chromatin research model organism. Dim-5 (H3K9 methyltransferase), Dim-2 (cytosine methyltransferase), adaptors, and heterochromatin protein1 (HP1) are essential elements of this complex (Lewis et al., 2010; Rountree and Selker, 2010; Smith et al., 2011). Any dysfunction in each component of this complex leads to DNA methylation disorders. HP1 deletion mutation (*hepAΔ*) did not show any cytological or morphological defect in *Aspergillus nidullans*, while extremely up-regulated SM gene clusters. Like this, other studies inhibiting methyltransferase complex decrease heterochromatin marks and induced SM production (Reyes-Dominguez et al., 2012).

5-azacytidine (5-AC) is an analog of the naturally occurring pyrimidine nucleoside cytidine and a DNA methyltransferase inhibitor. 5-AC is an effective tool to induce expression of silenced SM genes in fungi (Henrikson et al., 2009; Sun et al., 2012; Chen et al., 2013; Vasanthakumari et al., 2015; Kumar et al., 2016). In a study, *Muscodor spp.* treated with 5-AC produced new secondary metabolites ergosterol and xylaguaianol C. Gene expression researches showed the production of new metabolites in *Muscodor spp.* could be attributed to the activity of otherwise silent polyketide synthase genes (Qadri et al., 2017). 5-AC induced production of alternariol, altertoxin II, and other new compounds in *Alternaria sp.* (Sun et al., 2012). Production of new metabolites dendrolid E, G, and I by 5-AC treatment is also reported in *Dimorphosporicola tragani* (González-Menéndez et al., 2019). Also, 5-AC increased camptothecine production in *Botryosphaeria rhodina* fungal endophyte (Vasanthakumari et al., 2015). In most filamentous fungi, genes encoding independent steps of secondary metabolites biosynthesis, are clustered in the fungal genome. These clusters are synchronously regulated (Keller et al., 2005; Yu and Keller, 2005; Bohnert et al., 2010). Although hundreds of secondary metabolite biosynthesis genes are discovered in fungal genome sequencing, these genes are mostly silent and their secondary metabolites are unknown. DNA methylation and chromatin state regulation play a critical role in the overexpression of genes for SM biosynthesis. This makes the chance to use epigenetic modifier compounds to induce the transcription of SM genes (Yang et al., 2013).

As the cultivation of medicinal plants is subjected to geographical and seasonal variation, and lots of uncontrollable factors influence metabolite production, substituting another source of natural tanshinone producers seems essential. Therefore, the aim of the present study was to determine the possibility of tanshinone production by endophytic fungi isolated from *S. abrotanoides* and also investigate the ability of 5-AC to increase tanshinone production in fungal endophytes.

Materials and Methods

Plant material and fungal isolation

Pure fungal endophytes were isolated from roots of *Salvia abrotanoides* (Kar.) *Sytsma*. In previous study the ability of four fungal endophytes to produce tanshinone including to *Penicillium canescens*, *Penicillium nodositatum*, *Penicillium pinophilum*, and *Paraphoma radicina* was confirmed (Teimoori-Boghsani et al., 2020). These fourspecies from our pure isolates were cultured on malt agar medium. The first pure culture was used for inoculating liquid cultures. After a week, a piece of fresh hyphae from the first pure *P. pinophyllum* culture was subcultured to a new malt agar medium. This new culture was subcultured 3 times. The third subculture of *P. pinophyllum* was used to investigate the effect of generation on tanshinone production.

5-Azacytidine treatment

A section of fresh hyphae from a first pure solid culture of four species, and the third subculture of *P. pinophyllum* was transferred to 100 ml potato dextrose broth in 250 ml flasks. Liquid cultures were incubated on an orbital shaker with 100 rpm at 30 ºC. After a week, 5-Azacytidine (5-AC) was added to the flasks of the treatment group to reach 10 µM 5-AC in each flask (Vasanthakumari et al., 2015). The control group didn't receive anything in this step. After 20 days of treatment, fungal mycelium was filtered through a fine mesh and air-dried under low pressure. Five mg of dry mycelium were powdered, suspended in 5 ml ethyl acetate, and sonicated for 25 minutes at 40 ºC. The extracts were filtered 3 times through the Whatman paper. Extract in weighted vials was dried under evaporation with low pressure. Plant roots were washed and dried, then grounded. 5 mg ground roots were mixed with 5 ml ethylacetate in a tube. Tubes were sonicated for 40 minutes at 40 ºC and after were centrifuged. The supernatant liquid was transferred to a new weighted vial. This extract was dried overnight with low pressure. Four fungal

isolates that produced tanshinone were treated by 5-AC. This study was conducted as a factorial experiment based on completely random design with three replications. The generation effect on tanshinone production was investigated on the third subculture of *P. pinophyllum*. Also, 5-AC treatment was performed on the third subculture of *P. pinophyllum* to compensate generation effect.

HPLC analysis

Dried extracts of the previous step were solved in ethyl acetate and used as an HPLC sample. HPLC analysis was performed on a Knauer apparatus using a Eurospher 100-5 C18 chromatographic column (250×4.6 mm, 5 µm). The mobile phase consisted of methanol and H_2O (2% acid acetic) using the following gradient: 0 min, 70% methanol; 7 min, 70% methanol; 15 min, 100% methanol; 30 min, 100% methanol; 32min, 70% methanol, 35 min, 70% methanol. The injection volume was 20 µL for each sample and three injections were made to calculate the averages and standard deviations. The flow rate was 1.0 mL/min. The UV detection wavelength was 254 nm. Standard calibration was obtained with 1, 5, 6, 50, and 100 ppm cryptotanshinone concentrations. Also, four different concentrations of TA IIA (3, 5, 10, and 20 ppm) were injected as standard samples. The area under standard picks was estimated by EZ Chrom software. The regression equation and correlation coefficient were calculated in excel.

Statistical analysis

This experiment was performed using a factorial experiment based on a completely random design with five replications. Two-way analysis of variance (ANOVA) was performed via IBM SPSS V.23.0, and Duncan's multiple range test was performed to define the statistical differences at $P < 0.05$. The results were stated as the mean ± standard error (SE).

Result

5-Azacitidine effect on tanshinone production

HPLC analysis showed four species produced tanshinone in liquid culture. Cryptotanshinone and tanshinone IIA were produced by *P. canescens*, *P. nodositatum*, and *P. pinophilum*, while *Paraphoma radicina* only produced tanshinone IIA (Figure 1). In the control condition, the maximum amount of cryptotanshinone and tanshinone IIA (162.15±25.26 mg cry/g dw, 50.155±1.41 mg TIIA/g dw) was produced by *P. pinophilum* which is extremely more than the amount produced by plant root ($p<0.05$) (0.55±0.01 mg cry/g dw, 1.3±0.02 mg TIIA/g dw). The amount of cryptotanshinone produced by other endophytes was nearly similar to the amount produced by plant roots (Figure 2). 5-AC treatment made a big jump in T IIA production in *P. radicina* ($p<0.05$) (63.176 ± 0.13 mg TIIA/g dw), besides increasing T IIA production in *P. nodositatum* cultures ($p<0.05$) (Figure 3). the 5-AC treatment decreased T IIA and cryptotanshinone production in *P. pinophilum*. The amount of TIIA produced by 5-AC treated *P. nodositatum* was more than the amount produced by 5-AC treated and control cultures of *P. canescens* ($p<0.05$). In addition, the effect of 5-AC increasing TIIA production on *P. nodositatum* was more potent than on *P. pinophilum.* The amount of produced TIIA by *P. nodositatum* and *P. pinophilum* under 5-AC treatment was more than TIIA extracted from plant root ($p<0.05$).

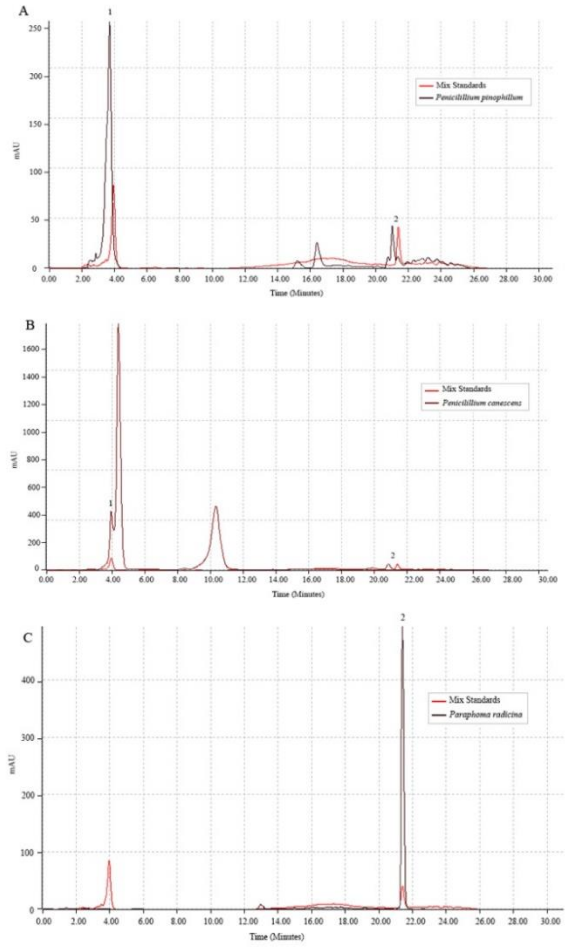

Figure1. HPLC chromatogram of fungal extracts over cryptotanshinone (1) and tanshinone IIA (2) standard picks.

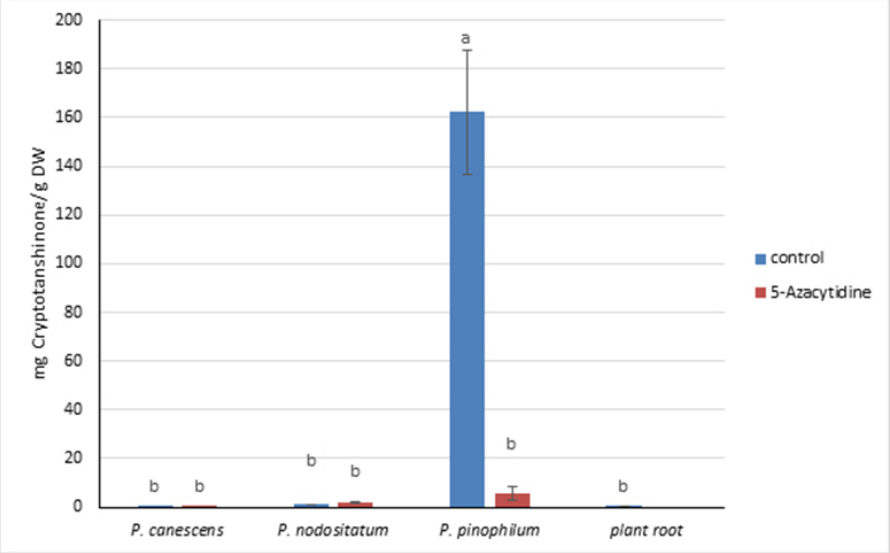

Figure 2. cryptotanshinone produced by the first subculture of fungal endophytes under control (cultures without 5-AC treatment) and 5-AC treatment. Means ± SE. Bars with similar alphabets are not significantly different (P<0.05)

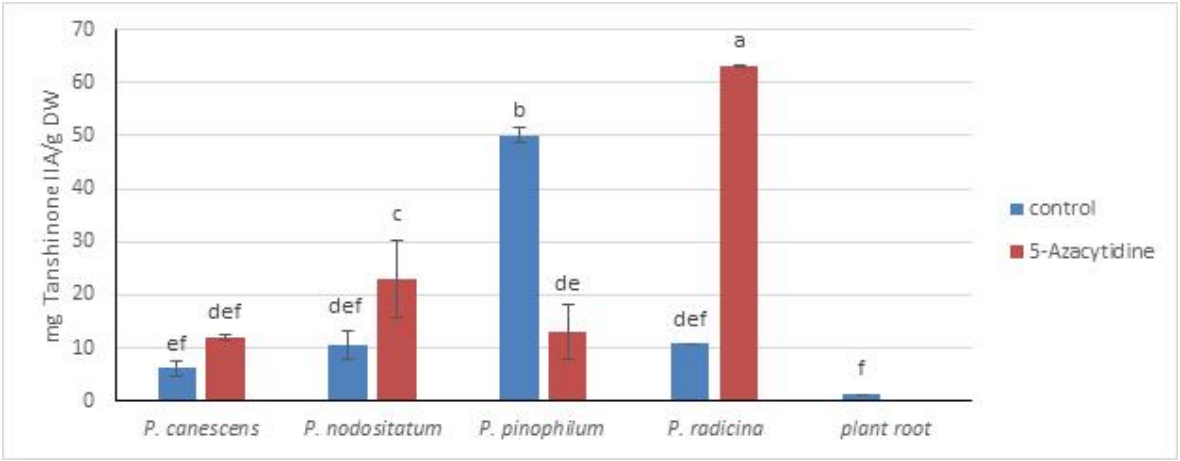

Figure 3. tanshinone IIA produced by a first subculture of fungal endophytes under control (cultures without 5-AC treatment) and 5-AC treatment. Means ± SE. Bars with similar alphabets are not significantly different (P<0.05)

The interaction effect between generation and 5-azacytidine on tanshinone production

After three subcultures of *P. pinophilum*, tanshinone production strongly decreased. Cryptotanshinone production dropped to a 100-fold lower amount. TIIA reduction amount was nearly 10-fold after three subcultures. 5-AC treatment significantly retrieved T IIA production in the third subculture of *P. pinophilum* (Figure 4). This treatment increased TIIA production to 3-fold more than the third control subculture. Cryptotanshinone produced by the third subculture of *P. pinophilum* under 5-AC treatment showed any significant changes (Figure 4).

Discussion

These results are in line with the results of Ming et al. (2012), who reported tanshinone IIA and I were produced using endophytic fungi (3.049 ± 0.001µg T IIA/g DW). Our results showed that *P. pinophilum* in the control culture produced an extremely higher amount of tanshinone IIA (50.155 ± 1.41 mg/g DW). Later studies focused on increasing secondary metabolite production in endophytes. In an experiment with *Trichoderma atroviride* a significant increase in the content of tanshinone I in plants treated with KAR1 (smoke-isolated butenolide) was reported(1.11 fold compared to the control) (Zhou et al., 2019). A mutant line of

Emericella foeniculicola produced a relatively high value of tanshinone IIA, a 1.46-fold increase in tanshinone IIA production compared with the wild type (Ma et al., 2011). Based on recent studies epigenetic modifiers are a successful method to bring SM production jump in microorganisms. The use of 5-AC possibly affects gene expression profile and significantly increases tanshinone production. Different *Penicillium* species were treated with 5-AC to increase SM production or gain new metabolites. Treatment of *P. citreonigrum* cultures with 5-AC induced production of sclerotiorin and seven other compounds (Wang et al., 2010). In cultures treated with 5-AC *P. funiculosum* produced two new diterpenoids with cytotoxic and antibacterial activities (Liu et al., 2014). This is the first report using 5-AC to improve tanshinone production in endophytic fungi. 5-AC treatment (10 µM) increased the content of TIIA in *P. pinophilum* third subcultures 3-fold more than the control culture. Also, TIIA production enhanced in *P. nodositatum* and *P. radicina* 5-AC treated cultures more than 2 and 5.8 times compared to the control cultures, respectively (p<0.05). Non-toxic concentrations of 5-AC induce hypomethylation through the depletion of cellular DNMTs (Griffiths and Gore, 2008). In this study, tanshinone biosynthesis genes are probably hypomethylated and upregulated under 5-AC treatment, which increases tanshinone IIA production. Determining the exact mechanism of tanshinone production enhancement needs detailed gene expression and metabolite profiling studies. Anyway using epigenetic modifiers is an easy and quick way to increase the production of pharmaceutical and valuable compounds in endophytic fungi.

Acknowledgments

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Conflict of Interests

The authors of this study declare that they have no conflict of interest.

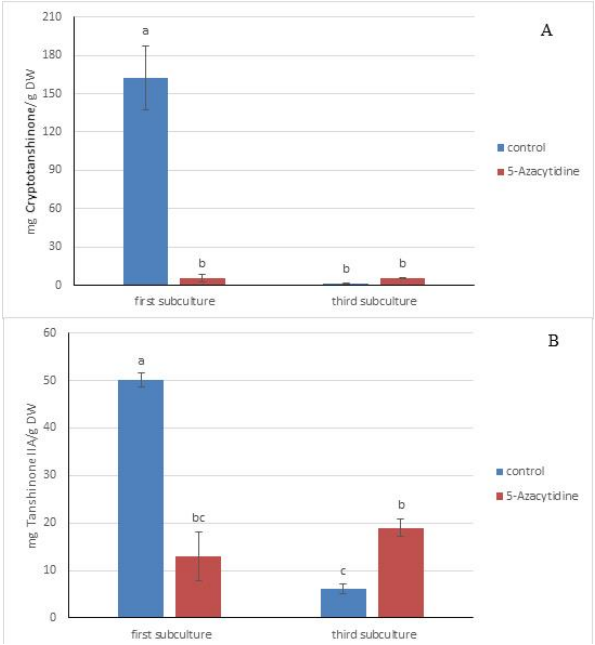

Figure 4. cryptotanshinone (A) and TIIA (B) production in first and third subcultures of *P. pinophilum* under control (cultures without 5-AC treatment) and 5-AC treatment. Means ± SE. Bars with similar alphabets are not significantly different (P<0.05)

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