Influence of Titania–graphene nanocomposite and coronatine on taxanes production and expression patterns of the key genes involved in taxol biosynthetic pathway in cell suspension culture of Taxus baccata

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Abstract

In this study, the potential effects of two elicitors of coronatine and TiO_2/graphene nanocomposite were evaluated on the expression of *txs*, *dbat*, *bapt* and *dbtnbt* genes, as well as taxanes production in cell suspension cultures of *Taxus baccata*. For this purpose, we studied the influence of 10 μMl^{-1} of coronatine and 30 μgl^{-1} of TiO_2/graphene nanocomposite along with phenylalanine and benzoeic acid at the concentrations of 0.003 mM and 1.0 mM, respectively. Our results indicated that the amounts of taxanes induced by TiO_2/graphene nanocomposite were upper than coronatine and control (42.47, 22.187, and 13.36 μgl^{-1} DW). The maximum amounts of baccatin III and taxol were detected at four days after treatment with TiO_2/graphene nanocomposite. The highest quantities of 10-deacetylbaccatin III and 10-deacetyltaxol were detected in day 16 after treatment with TiO_2/graphene nanocomposite and the control, respectively. The amount of cephalomannine get raised gradually with TiO_2/graphene nanocomposite. Meanwhile, TiO_2/graphene nanocomposite increased the relative gene expressions *TXS* and *DBTNBT* than Coronatine. The uppermost amount of *TXS* expression was observed 4 days after application of two elicitors. The *DBAT* gene exhibited the lowest gene expression among the four genes studied. The minimum and the maximum level of *BAPT* expression was detected after treatment with coronatine and TiO_2/graphene nanocomposite at day 4, respectively. The highest expression of *DBTNBT* occurred after treatment with TiO_2/graphene nanocomposite at day 2, whilst treatment with coronatine had no effect. In conclusion, TiO_2/graphene nanocomposite was more effective elicitors to induce production of taxanes and gene expressions levels during experiments as compared with coronatine and control.

Introduction

Paclitaxel has been considered as one of the most effective anti-cancer drug since its confirmation by FDA in 1992 and 1994 as an important drug compound for the treatment of ovarian and breast cancers, respectively. This plant-based anticancer drug was initially extracted from the inner bark of yew tree (*Taxus brevifolia*), but owing to small amounts of the compound and tree harvesting, and subsequent environmental concerns, some alternative sources were gradually introduced including plant callus and suspension cultures (Zwawiak & Zaprutko, 2014).

Cancer is one of the major reason of death worldwide, having caused an average 5.2 million deaths each year in the past decades (GLOBCAN 2012; Pandi et al. 2013). Prediction of future cancer incidence and mortality burden worldwide from 2018 up until 2040, indicated that the case of cancer would increase from 18.1 to 29.5 million cases (WHO & http://gco (Global Cancer Observatory(GLOBCAN)). Estimated data stated that the selling of taxol-derived drugs in the world from 2019 to 2025 would rise from 90 to 140 billion US dollars. Production of taxol in 2017 was estimate as 2600 kg in the world (www.reportsweb.com; www.research & market.com).

The yew tree (*Taxus* spp., Taxaceae; 2n = 2x = 24; 29) is a slow-growing, evergreen, resin-free gymnospermous shrub (Wang et al. 2011). The genus *Taxus* spp. is taxonomically included nine species (Wang et al. 2011), among them *T. baccata* is existed in the north regions of Iran (Nasiri et al., 2016). *Taxus* species produce an impressive array of taxanes, a group of diterpenoids with a taxane skeleton as their characteristic constituents.

Taxane acts as a microtubule disruptor, connecting to microtubules and blocking the cell cycle by inhibiting depolymerization of microtubules in the metaphase- anaphase transition (Gallego et al., 2015 and Sajjad *et al*., 2012). To date, more than 550 taxanes have been identified. Paclitaxel (Taxol A, Taxol@), alongside baccatin III

(BAC III) and 10-deacetylbaccatin III (10-DAB III) as the two pivotal precursors for the practical semi-synthesis of taxol and taxotere have been regarded as the most important taxanes ever discovered (Zhao et al., 2015).

The first step of taxol biosynthesis is the cyclization of GGPP to the taxa-(4,5), (11,12)-diene and this reaction catalyzed by taxadiene synthase. Several enzymes play important role in the synthesis of taxol to form baccatin III, including taxadiene-5 α hydroxylase (T5 αH), taxadiene-5 α-ol-O-acetyltransferase (TDAT), dependent Hydroxylase of cytochrome P450, taxoid 14ß-hydroxlase (T14ßH). In addition, hydroxylation, oxidation and the epoxidation follows at certain positions. Also, P450 cytochromes involved in five additional oxygenase (Kaspera & Corteau, 2006). The next enzymes including 2 α-O-benzoyl transferase (DBT), 10-deacetyl-baccatin III-10O-acetyl transferase (DBAT), PAM enzyme, C-13-phenylalanoyl-CoA transferase BAPT ,3 -N-debenzoyl-2 -deoxytaxol N-benzoyl transferase (DBTNBT) lead to produce taxol (Malik et al., 2011; Onrubia et al., 2010).

Callus cultures of *T. caspidata* and *T. Canadensis* were obtained from different tissue explants. Callus derived from stem segments exhibited the best growth in B5 medium supplemented with 2–4,D and kinetin. For avoiding of darkening, cysteine, ascorbic acid, PVP and activated charcoal were used by Fett-neto et al.,1992. Brunakova (2004) were used 6 mg/l of 2–4,D and 0.5 mg/l of Kinetin for callus induction of *T. baccata*. The cells preserved in Gamborg's B5 medium (Gamborg et al., 1968) supplemented with 1.5% PVP and 3 mg l^{-1} 2–4,D and 0.5 mgl^{-1} kinetin in 22 ± 2 ºC under dark condition. Furthermore, Wickremesinhe et al., (1993) were used B5 and 2X B5 vitamins with 2–4,D, NAA, and IBA plus kinetin with sucrose, glucose plus fructose and casein hydrolysate. The yield of taxol production ranged between 0.1–13.1 $mgKg^{-1}$ DW.

Elicitors are chemical or bio-factors from different sources including abiotic and biotic elicitors, that are able to induce and trigger signal transduction in target cells (Zhao et al., 2005). Jasmonates play an important role in response to wounding and production of secondary metabolites (Onrubia *et al*., 2013). *Pseudomonas* spp. produces a wide spectrum of phytotoxic compounds. The most well characterized bacterial phytotoxins produced by *Pseudomonas syringae*. These phytotoxins are mostly identified with two symptoms, a primary symptom, chlorosis (coronatine, phaseolotoxin, and tabtoxin) or necrosis (syringomycin and syringopeptin). Coronatine (COR) is classified as polyketide, and polyketide synthesis is related to fatty acid biosynthesis (Uppalapati et al., 2005 & Bender et al., 1999). Coronatine acts like a molecular mimic of jasmonate isoleucine-conjugated and produce JA-Ilu and triggered virulence effect via activation the jasmonic acid signaling pathway (Onrubia et al., 2013).

The capacity of Nano-titania (TiO_2) with unique physicochemical properties take much attention for biomedical applications. Furthermore, graphene sheets (GS) are considered as a new potential material in biology; because of high surface area, mechanical stiffness, and bacterial inhibition. Even though, the addition of reduced graphene oxides (graphene) is a new potential material with enhanced mechanical property, bioactive nature and cytocompatibility via promoting better cell adherence (Kandiah et al., 2014). The influences of coronatine and metyl jasmonate (Mej) on *T. media* cell culture were studied by Onrubia et al (2013). They were used two stages of cell culture for growth and production of secondary metabolites. The effect of two elicitors vanadyl sulphate (VS) and metyljasmonate (Mej) were investigated for identifying taxanes patterns and transcripts profile of two genes of *txs* and *bapt* in *T. baccata* cell line by Onrubia et al., (2012). Kajani et al., (2012) investigated five types of culture media including, B5 (Gamborg), MS (Murashige and Skoog, 1962), WPM (Woody Plant,), Schenk and Hildebrandt, and DKW (Driver and Kuniyuki, 1984) on production of intra and extra cellular taxanes. Their results showed that DKW media increased significantly the yield of taxanes from other investigated media.

In this study, the potential effects of two elicitors of TiO_2/graphene nanocomposite and coronatine were examined on taxane production and expression levels the key genes **involved in taxol biosynthetic pathway** from cell suspension culture of *T. baccata*.

Material And Methods

Cell suspension culture

Plant material was sampled from *T. baccata* grown in the Botanic garden located in the College of Agriculture, University of Tehran, Karaj, Iran. Perennial and annual branches were used for sampling. At first, shoots were submerged under tap water and brushed. Then, they were immersed in 70% ethanol for 2 minutes. Explants rinsed with deionized water once again, then shacked in 2.5% sodium hypochlorite for 15 minutes. After washing for another 10 minutes, it washed with ddH_2O. We used B5 media in the glass containing 60 ml of solid media supplemented with 8 gl^{-1} of agar. For callus induction, both 2-4-D (6 mgl^{-1}) and Kinetin (0.5 mgl^{-1}) were used, and PVP (1.5%) was utilized for avoiding of darkening. The jars transferred in dark room at 23 ºC. After one week, the samples were evaluated for bacterial and fungal contamination. Explants from perennial shoots had more infection, but for achieving good result with increasing the time and duration of sterilization by sodium hypochlorite, only the older branches were sampled at the last stage. After producing the fresh and productive callus, subcultures were performed every three weeks. The suspension culture was performed in 20 ml of liquid B5 supplemented with 3mgl^{-1} of 2–4,D and 0.5mgl^{-1} of kinetin. We used 1gr fresh and white callus in 20ml of media for evaluating of their growth rate. At the first week 9 out of 20 Erlenmeyers harvested, then their fresh and dry weights were measured after lyophilization using freeze dryer for HPLC analysis. For the next week, we harvested 5, 4, 3, 2 and 1 erlenmeyers respectively for the second, third, fourth and fifth week before subculture. The chart of growth curve with two elicitors was drawn with coronatine 10µM and titania graphen nano-composite. The amounts of applied elicitors were based on the previous researches.

Extraction And Detection Of Taxanes

Extraction of cell suspension culture was performed based on the method descripted by Fett-neto et al (1992), and Han et al (1997) with minor modifications. The freeze-dried and grinned cells were solved in methanol, vortexed thoroughly and sonicated for 30 min with ultrasound at 40 KHz, incubated then at room temperature for 16 h. Samples were centrifuged in 25000 g for 20 minutes. After that supernatant was recovered. The methanol extract combined with equal volume of dichloromethane and water (3:1 v/v) in order to separate two phases (organic and aqueous phases). The mixture was poured in the decanter funnel after three time washing with dichloromethane and deionized water (3:1 v/v), the organic phase returned rotary to evaporate at 25 ºC, the solid phase was resolved in the acetonitrile. The solvent was incubated in -20 to precipitated taxenes for 3 days. After this, HPLC was performed. Isolation was carried out with analytical column Eurospher 100-5 C18. For obtaining and comparing data, the data implemented with Melanium software and the evaluation of chromatography were performed with using reversed phase matrix (5µl) waters system. The rinsing was done in gradient system with acetonitrile as an organic phase (solvent A) and deionized water (solvent B) with 1ml/min flow rate. The wavelength of peaks monitored in 230 nm. Injection volume was 20 µl at 25 ºC with two technical replications.

Rna Extraction, Cdna Synthesis, Pcr And Qrt-pcr

RNA was extracted using the protocol of Zarei et al (2012). *DNase I* treatment was performed. Nanodrop and 2% agarose electrophoresis was performed to ensure RNA quantity and quality. In order to optimize annealing temperature of the primers, PCR reaction was performed with vivantis kit (http://www.vivantechnologies.com) in a total volume of 25 µl. cDNA synthesis was implemented in two step reactions. The first step including oligo dt (4µM), random hexamer primer (0.000005 µg/µl), dNTP Mix (1mM) total extracted RNA (120ng/µl) in total volume of 10 µl. The second step of cDNA synthesis contained M-Mulv Reverse transcriptase (200 Uint, 10U/µl) and 10x buffer M-Mulv (2µl) in a total volume of 10 µl. To ensure the quality and quantity of cDNA, gel electrophoresis and Nanodrap were used. The primers sequences were according to Nasiri et al (2016), but *18s* primers were designed using primer3 implemented in Geneious (Table 1). For gene expression, Real Time PCR was performed by using Abi tools (http://www.appliedbiosystems.com). qPCR reaction was performed with 1.0 µl cDNA with 300 ng/µl concentration, 0.2 µl of each Primer, 5 µl of ampliqon SYBR green 2x high ROX Cyber green master mix PCR 2x, 3.6 µl of dH2O nuclease free contained total volume of 10 µl.

Table 1
The primer sequences used in amplification and expression of *TXS, DBAT, BAPT*, and *DBTNBT* genes in *T. baccata*

Locus	Primer sequences	Length of amplified sequences (bp)	Annealing temperature of primers (°C)	Accession NO.	Reference
TXS (F)	5´-CCGTGTACCCTACAACCAATAC-3´	118	57	AY424738.1	Nasiri *et al*., 2016
TXS (R)	5´-TTGTTAGTCGCCAGCTCAAG-3´				
DBAT (F)	5´-CTCTCCACCCTTGACAATCTAC-3´	119	57	KC571283.1	Nasiri et al., 2016
DBAT (R)	5´-GAGAGAGCCTGCCGAATTAC-3´				
BAPT (F)	5´-TGTGGGAGCGAATGTGTATG-3´	137	57	JQ029681.1	Nasiri et al., 2016
BAPT (R)	5´-CTAGCTTCACCAGTTCTCTATTCC-3´				
18S(F)	5´-GGAGTATGGTCGCAAGGCTGAAAC-3´	61	57	DQ478811.1	Designed by authors (18S *taxus*)
18S (R)	5´-CTCAATCTGTCAATCCTCACTATGTCTGG-3´				

Data analysis

Statistical analysis for HPLC result, was carried out using SPSS ver. 16 and mean comparisons run at 5 percent probability level was accomplished using Duncan method. Data analysis for gene expression of key genes of *BAPT*, *DBAT*, *TXS* and *DNTNBT* was performed using REST software.

Results And Discussion

Cell culture condition of T. baccata

B5 medium supplemented with 6 mgl^{-1} of 2-4-D and 0.5 mgl^{-1} of Kinetin were used for induction of callus. The fresh and white calli obtained after 6 weeks (Fig. 1).

Cell Growth Curve And Elicitation

The cell growth curve was drawn for determination of exponential time for applying the elicitors in media (Fig. 2). Two elicitors for treatment of cell suspension including 10 µM coronatine and 30 µM TiO_2 for elicitation were used. The harvesting of cells carried out after 2, 4 and 16 days from treatment due to Onrubia et al., 2013 research. The elicitors were added at the beginning of stationary phase. The viability curve achieved based on cell growth after treatment with two elicitors, but for coronatine, based on Onrubia et al., 2013, 1 µM was used and we ought to use this concentration (the result not shown), but the production of taxanes was very low, so we used 10 µM of coronatine.

Viability of cells were measured after treatment based on dry weight after passing 16 days.

60 gl^{-1} of fresh callus were used for induction in cell suspension culture and the dry weight was 3.634 gl^{-1} and for TiO_2 was 5.281 gl^{-1} and for control was 4.987 gl^{-1}.

The chart indicated that during the first and second weeks, cellular weight changes was very low but during the third week cell weight increased, this point is the best time for adding elicitors and increasing taxanes production (Fig. 2).

Immobilization

Immobilization is one of the most important strategies for increasing cell production and secondary compounds. Immobilized cells have many advantages over free suspended cells as immobilization provides higher cell concentration per unit of volume, higher cell–cell contact and protection from liquid flow. Plant cells are immobilized using different gels including alginate, carrageenan, polyacrylamide, agarose, polyurethane foam, and hollow fiber (Malik et al., 2011). Bentebibel et al (2005) used calcium alginate for immobilization of the taxol and BAC III producing cells of *T. baccata* and their results identified that immobilization enhanced the production of taxol and BAC III compared to free cells. The revenue of alginate-entrapped cells at day 24 was 5.5 fold higher than free cells. In this study, the potential of TiO_2/graphene nanocomposite for immobilization of cells were studied. chemical reaction and bonding between TiO_2 and nano graphenes sheets provided very powerful electrical and mechanical connection inside the hybrid. This capacity generates a new candidate material and reinforced mechanical property, bioactive character and cell compatibility via improving better cell adherence. The structure of TiO_2/graphene nanocomposite is in the picture (Fig. 4).

In our study this ability of TiO^2/graphene nanocomposite applied. Figure 3 shows the dry weight of cells after inducing TiO_2/graphene nanocomposite get rise 1.3 fold as compare with control and 1.89 fold as compare with coronatine.

The Amount Of Taxenes Changes During Cell Growth Curve

The amount of 10-DAB III from the second to fifth weeks increased simultaneously along with a decrease in BAC III amount. Also, 10-deacetyltaxol was detected in the last step of cell growth curve, but taxol was traced at this time. Viability of cells based on dry weight indicated that the cell biomass decreased during elicitation after treatment with TiO_2/graphene nanocomposite and coronatine as compare with control. where coronatine had more negative effects. It may be related to graphene and composite structure that supported the cells. Taxanes content in cell growth curve in the figure

Hplc Quantification Result

Quantification of taxanes including 10-DAB III, BAC III, 10-deacetyltaxol, cephalomannine and taxol established by HPLC. The results (Table 2) displayed that, the BAC III content increased gradually until day 4 (3.8 and 7.69 $\mu g l^{-1}$ DW) after treatment with nano titanium graphene composite and then decreased until day 16 (3.49 $\mu g l^{-1}$ DW) (Fig. 6a). The maximum amount of 10-DAB (9.17 $\mu g l^{-1}$ DW) obtained in day 16 after elicitation with nano titanium graphene composite (Fig. 6b). 10-deacetyltaxol production increased until day 4 by coronatine treatment (Fig. 6c). Quantification of taxol (1.35 $\mu g l^{-1}$ DW) increased in day 4 after treatment with nano titanium graphene composite as compared with the control (Fig. 6e). In addition, cephalomannine in treatment with nano titanium graphene composite get rose from day 2 until day16 after application of elicitor 0.605, 1.46 and 1.905 $\mu g g^{-1}$ dry weight, respectively (Fig. 6d). Totally, the amount of taxanes was 42.47 after treatment with nano titanium graphene composite.

Table 2
Variance components related to taxanes in *T. baccata* after application of two elicitors of TiO_2/graphene nanocomposite (30 $\mu g l^{-1}$) and coronatine (10 $\mu M l^{-.31}$)

Source of variation	degree of freedom	Mean of squares				
		BAC III	10-DAB III	10-deacetyltaxol	cephalomannine	Taxol
Treatment	2	19.198**	31.3**	0.41**	3.494**	1.59**
Day	2	7.263**	6.523**	0.642**	0.289**	0.348**
Interaction	4	4.14**	4.942**	1.057**	0.289**	0.133**
Error	9	0.096	0.144	0.002	0.009	0.018
CV %		10.5	8.6	13.5	18.1	25.6

Coronatine treatment had the highest amount of taxanes in day four and the total taxanes was 22.187 $\mu g l^{-1}$ DW. Overall, the treatment with TiO_2/graphene nanocomposite influenced yield of taxanes production more than coronatine. Variance comparison demonstrated that the application of two elicitors had positive effect on production of taxanes. Our result based on time exposure.

Expression of TXS, DBAT, BAPT **and** DBTNBT **genes in** Taxus baccata **L.**

Gene expression changes in *T. baccata* L. indicated that gene expression of *TXS* in the second day after TiO_2/graphene nanocomposite treatment in 30 $\mu g l^{-1}$ concentrations, got rise to 29.82 fold (treatment with coronatine gene expression of *TXS* in compare to control decrease and this gene down regulated relative to control, especially in the second day). The rate of *DBAT* gene expression decreased in the second day after treatment with both elicitors and for coronatine treatment declined more than TiO_2/graphene nanocomposite. In the day 4, after treatment with both elicitors, the rate of gene expression of *DBAT* get rise, as this increased 1.42 and 0.8 fold, respectively with coronatine and TiO_2/graphene nanocomposite.

The gene expression of *BAPT* in both coronatine and TiO_2/graphene nanocomposite, demonstrated downregulation and upregulation in the second and fourth day after treatment, respectively. However, it increased more in TiO_2/graphene nanocomposite than coronatine, 10.18 as compared with 1.72.

The expression of *DBTNBT* in *T. baccata* L. detected only for treatment with TiO_2/graphene nanocomposite, indicating that this elicitor has high capacity for inducing the genes that placed at the end of taxanes production pathway.

According to the expression of four evaluated genes, it could be illustrated that without up regulation of *TXS* for treatment with coronatine, *DBAT*, BAPT expressed in response to this elicitor, their mRNA transcripts increased, and their metabolites are raised. In treatment with TiO_2/graphene nanocomposite *TXS* expression get raised 4 days after treatment and its related metabolite 10-deacetylbaccatin increased more than coronatine treatment. Furthermore, when this basic metabolite gets rise by treatment, it would be induced the upstream gene to consume the metabolite, also the mRNA transcript levels of *DBAT* and *BAPT* and *DBTNBT* genes with TiO_2/graphene nanocomposite application and subsequently their metabolites could be increased. The successful treatment for enhancing production of taxol have been studied with Onrubia et al., 2010, including supplemented media with metyljasmonate on the cell lines obtained from *Taxus* spp.

Anyway, the effect of elicitors in molecular aspect evaluated rarely, and subsequently the control of taxol biosynthesis pathway did not identify yet. Only Onrubia et al., (2010) demonstrated that after treatment with metyljasmonate up regulation of gene encoding *TXS* occurred, and in a low level of the enzymes that located at the end of taxol pathway are coded. According to Onrubia et al., (2010) the responses of cellular culture to the elicitor not only depends on the elicitor such as type, concentration, exposure time, but also depends on species and cell line developments. Influence of metyljasmonate and coronatine evaluated in *T. Media* by Onrubia et al. (2013), for detecting elicitors mechanism on the biosynthesis of taxanes. According to their results, expression analysis exhibited that the *txs*, *t13oh*, *t2oh*, *t7oh*, *dbat*, *pam*, *bata* and *dbtnbt* genes were not equally induced by the elicitors. Genes encoding enzymes participated in the constitution of the polihydroxylated hypothetical intermediate (TXS, T13OH, T2OH, T7OH) and the *PAM* were higher effected than those encoding enzymes catalyzing the last steps of the Px biosynthetic pathway (*DBAT*, *BAPT* and *DBTNBT*). According to their studies, day 2 and 4 had the maximum expression in all studied genes. Total taxane yields in the cell suspension culture was remarkably effected by two types of elicitors, rising from 8.14 $mg l^{-1}$ in control conditions to 21.48 $mg l^{-1}$L (day 12) with MeJ and 77.46 $mg l^{-1}$ (day 16) with coronatine.

Onrubia et al. (2013) implemented elicitations after 14 days on cell culture. Their results displayed that the yield of total taxane with MejA was 21.48 $mg l^{-1}$ (day 12) and with coronatine was 77.46 $mg l^{-1}$ (day 16). The gene expression indicated that the genes located at the beginning of pathway induced more than the genes at the end

of pathway *(DBAT*, *BAPT* and *DBTNBT*). Their results indicated that Mej treatment is able to increase the expression of two studied genes, while VS just was able to increase *bapt* gene expression. Also Mej activated baccatin III and taxol production thoughVS just enhanced taxol yeild. On the other hand, VS could not influence the expression of *txs*, that located at the beginning of pathway (Onrubia et al., 2010).

Conclusions

HPLC results and gene expression indicated that the expression of *TXS*, *DBAT*, *BAPT* and *DBTNBT* after treatment with TiO_2/graphene nanocomposite at day 2 and 4 after application, except in day four in expression of *DBAT* that had descending rate, all of them had ascending rate. The expression of *TXS* in day 4 as compared with control increased and its expression was more than control. The amount of 10-DAB in day 2 was higher than day 4 although *TXS* expression was in low level comparing to control. At day 4, the expression of *TXS* was very high, and it caused to produce 10-DAB, the amount of 10-DAB decreased because of consuming it by expression of *DBAT* and it caused to convert it to BACIII that it placed in downstream pathway. Expression of *DBAT* in day 2 was low, but in day 4 after application of TiO_2/graphene nanocomposite get rise. Expression of *BAPT* from day 2 to day 4 increased and in treatment with TiO_2/graphene nanocomposite was more than coronatine and it indicated that the producing of taxanes that placed downstream of gene expression pathway must be increased and the intermediate taxans such as 10-deacetyltaxol, cephalomannine and taxol should be get risen. The expression of *DBTNBT* gene in day 2 and 4 after application of TiO_2/graphen nanocomposite occured just in it and it not be induced in coronatine. Then, the amount of this taxanes after BACIII was not very high in coronatine treatment. The expression of *DBTNBT* in day 4 decreased as compared with day 2 and it caused to produce taxol in day 4 comparing to day 2 and day 16. The expression of *DBTNBT* decreased from day 2 to day 4, and the amount of taxol from day 2 to day 4 increased, but it decreased to day 16 although the amount of cephalomannine increased because of declining in the expression of *DBTBNBT*.

Declarations

Statements and Declarations:

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Competing Interests

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Figures

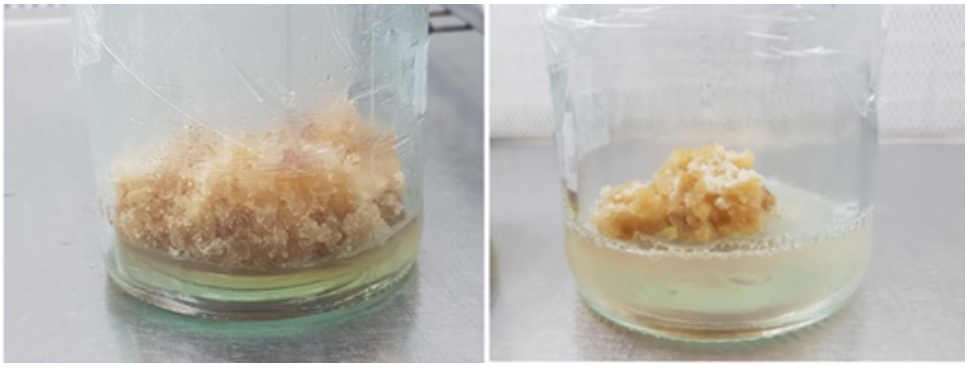

Figure 1

Fresh and white callus achieved from *T. baccata* L. in B5 media (the callus after 12 weeks was shown)

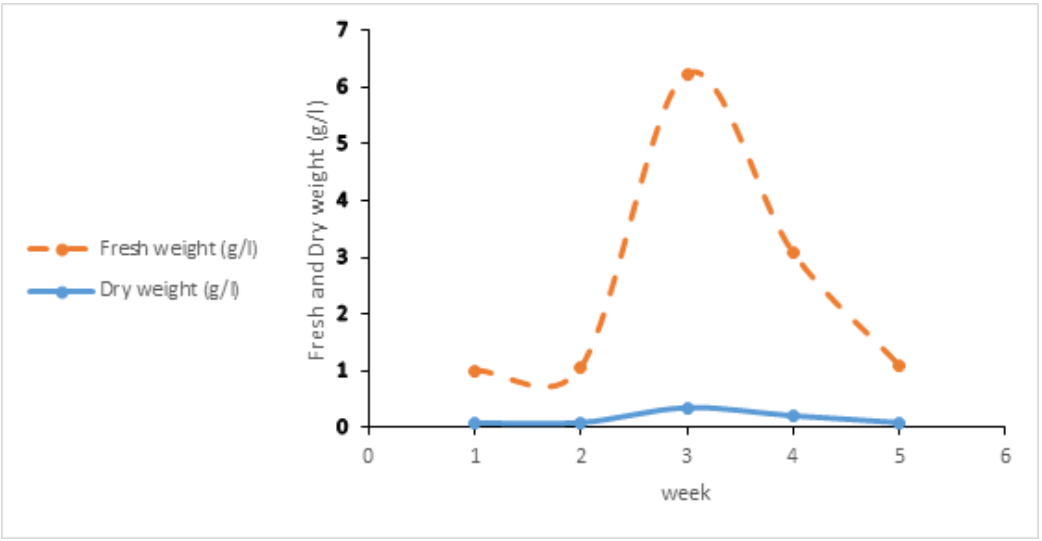

Figure 2

cell growth curve in terms of fresh and dry weight

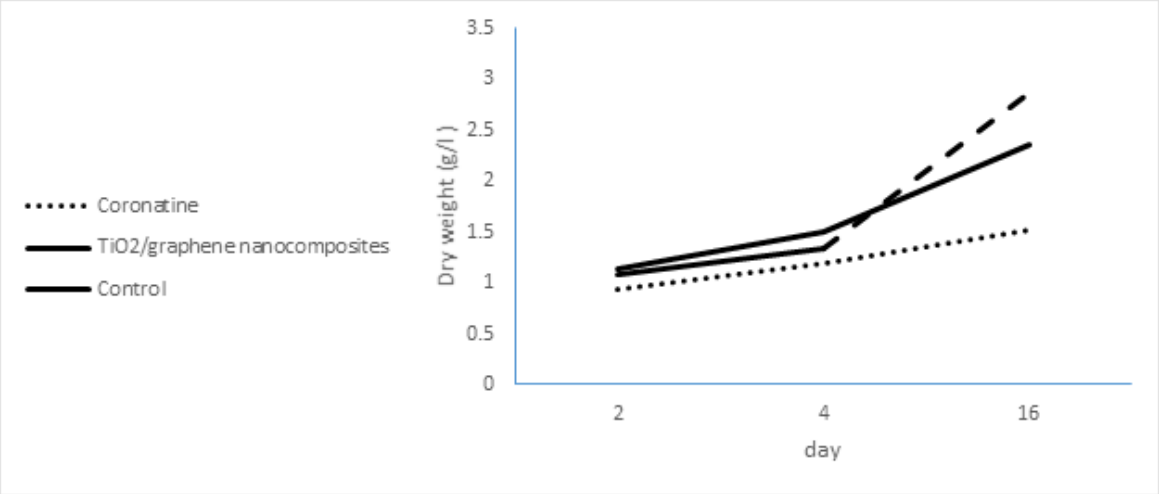

Figure 3

Cell growth after treatment with two elicitors and Control condition

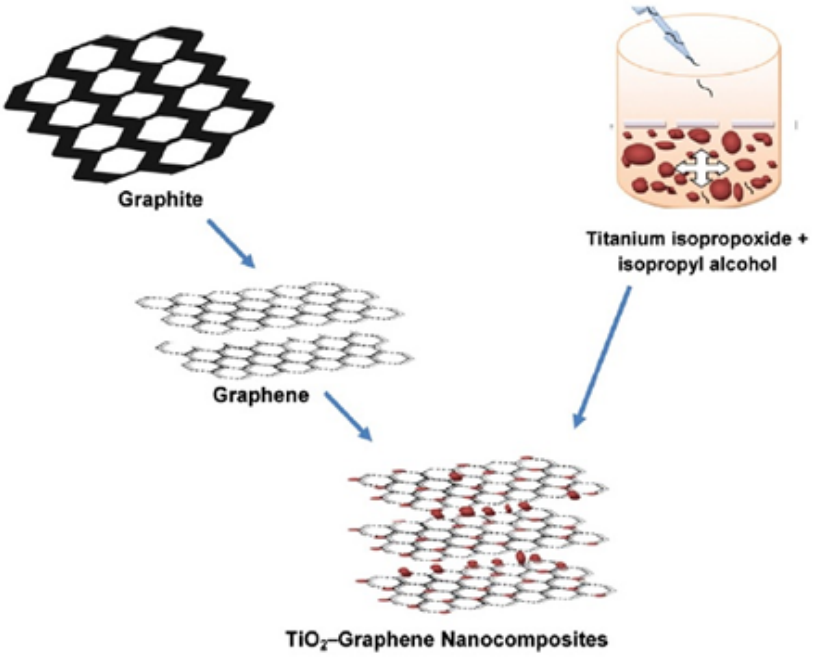

Figure 4

the formation of TiO_2-graphene nanocomposite (physical structure) (Kandiah et al, 2014)

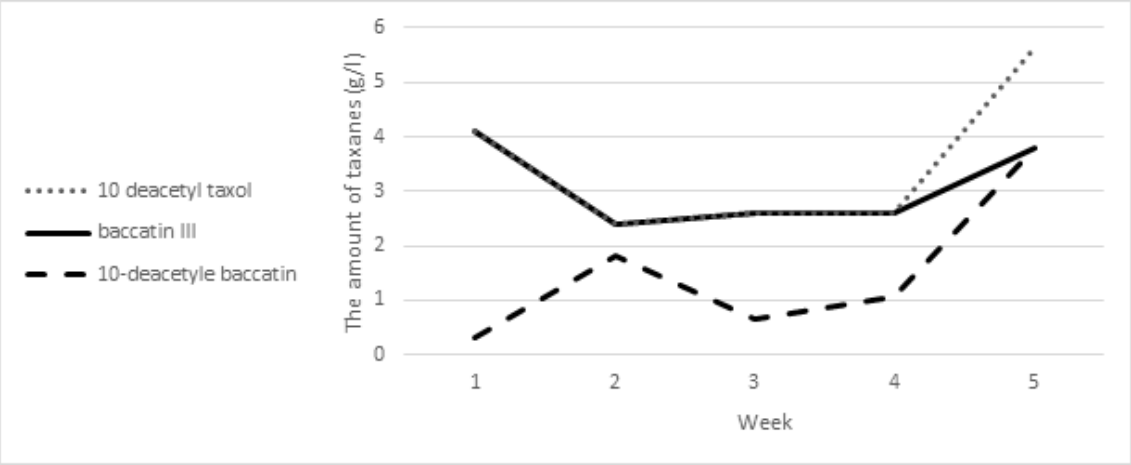

Figure 5

taxanes content in the cell growth curve with fresh and white *T. bacata* calli in 5 weeks, in terms of µg/l cell dry weight

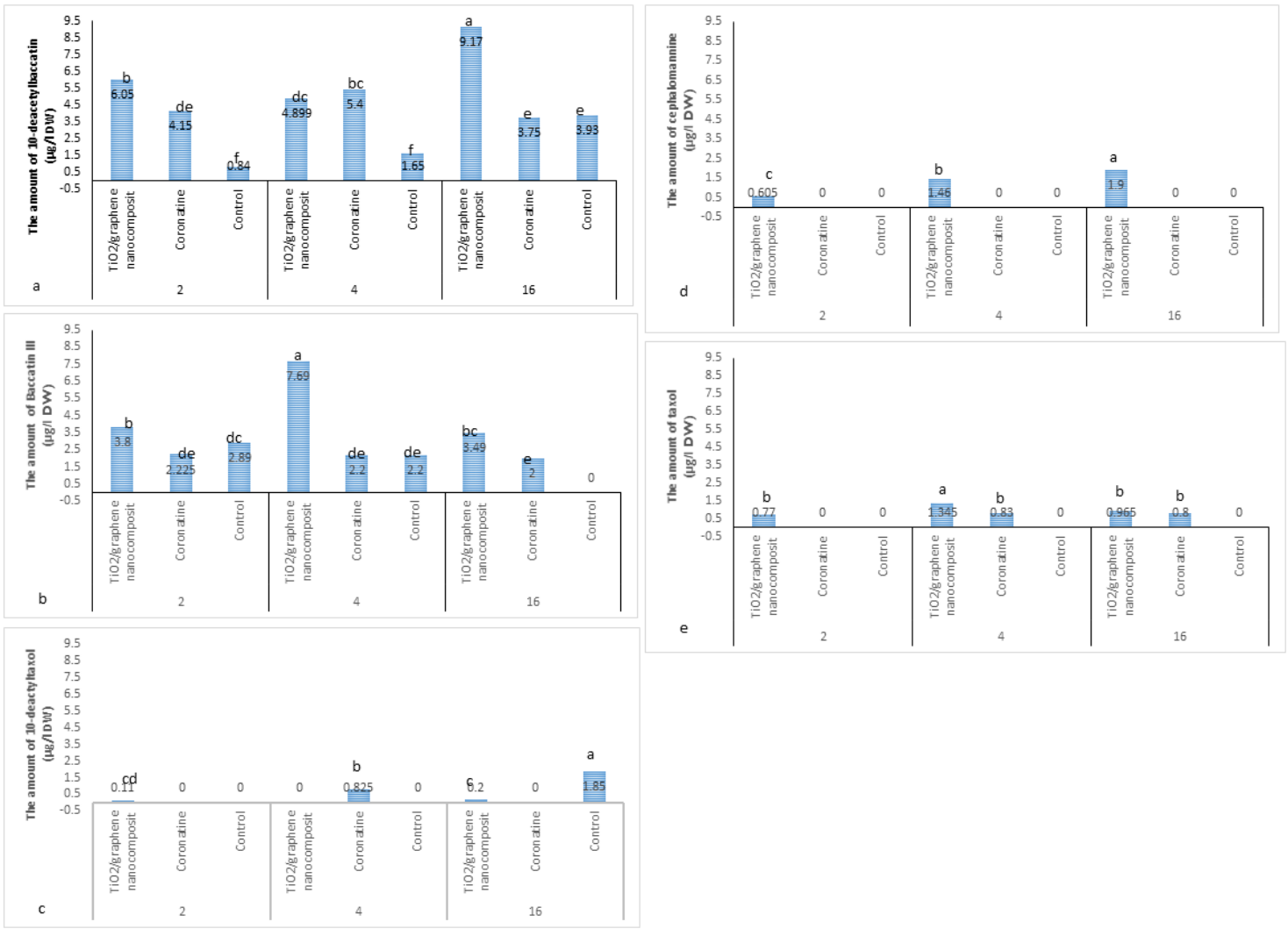

Figure 6

The changes of taxanes amount after treatment with TiO_2/graphene nanocomposite, coronatine and control after 2, 4 and 16 days, a) 10-deacetylbaccatin, b) baccatin III, c) 10-deacetyltaxol, d) cephalomannine, e) taxol