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Application of Thidiazuron (TDZ) for *in vitro* multiplication of yarrow (*Achillea millefolium* L.) and profile of volatile compounds

Ivan Caldeira Almeida Alvarenga*, Sâmia Torres Silva, Suzan Kelly Vilela Bertolucci, José Eduardo Brasil Pereira Pinto, Fernanda Ventorim Pacheco

Laboratório de Cultura de Tecidos e Plantas Medicinais, Departamento de Agricultura, Universidade Federal de Lavras, Lavras, Brazil

***Corresponding author: pytchoagro@yahoo.com.br**

Abstract

This study evaluated *in vitro* multiplication and volatile production of yarrow (*A. millefolium* L.) a medicinal plant, with different Thidiazuron (TDZ) concentrations. Explants with 10 mm length were inoculated into flasks containing 40 mL of Murashige and Skoog (MS) medium supplemented with 0.25, 0.50, 0.75 and 1.00 mg L^{-1} TDZ, and the control without the regulator. The experimental design was completely randomized with five replicates per treatment. At 45 days of culture, the number of shoots, shoot length and dry mass of shoots were evaluated. It was also checked the changes of the volatile constituents of leaves, in all treatments and plants within 30 and 75 days in MS free-hormone medium. The volatiles of leaves were analyzed by headspace-GC/MS. The application of 0.75 mg L^{-1} of TDZ showed the best combination of variables number, length and dry mass of shoots. Regarding volatile compounds, 32 constituents were identified, and the major constituents in all treatments were sabinene, 1.8-cineole, borneol, β-caryophyllene and β-cubebene. It was observed significant changes in the profile, number and contents of the constituents with presence of TDZ.

Keywords: tissue culture, yarrow, *headspace*-CG/MS, plant growth regulator, essential oil.
Abbreviations: TDZ_Thidiazuron, BAP_6-benzilaminopurine, CG/MG_gas chromatography/mass spectrum, MS_Murashige e Skoog medium, IP_initial plantlets, FP_final plantlets, NS_number of shoots, DMS_Dry mass of shoots.

Introduction

In vitro propagation of medicinal plants is widely used, quickly obtaining a large number of identical plants, with phytochemical and sanitary quality (Sivanesan et al., 2010). For the success of an *in vitro* culture, protocols specific to each species are necessary, using different culture media, salt concentrations and plant growth regulators. Thidiazuron (TDZ) is a plant growth regulator widely used in tissue culture and promotes cell division and elongation (Murthy et al., 1998). It operates in the regeneration and proliferation of meristems and, in combination with other regulators, can be used for the formation and maintenance of *callus* (Kokotkiewicz et al., 2012). TDZ has been successfully used for the propagation of several medicinal plants, such as *Curcuma longa*, *Ocimum basilicum*, *Vitex trifolia*, *Poupulos* spp. *Harpagophytum procumbens*, where the increase in the number and length of shoots is observed when there is an increase in concentrations of the regulator (Ahmed and Anis 2012; Naz et al., 2012; Grabkowska et al., 2014; Prathanturarug et al., 2005). Further advantage observed in the use of TDZ on proliferation of medicinal plants is the maintenance of genetic stability, important for obtaining plants *true-to-type* and germplasm conservation (Faisal et al., 2014). In general, studies with TDZ are related only to plant multiplication; however, its effect on the production of secondary metabolites is still poorly studied. As examples may be cited the increase of the silymarin content in regenerated plants *Sibylum marianum*, from explants multiplied with TDZ and the increased of phenolic compounds and antioxidant activity of *Artemisia absinthum* callus, under the regulator action. Both studies deal with constituents if the enzyme PAL (phenylalanine ammonia liase) it is the key enzyme in the biosynthesis process (Khan et al., 2014; Ali and Abasi 2014). TDZ can also increase the activity of terpene synthases enzymes, having a direct effect on the metabolism of terpenes, which is responsible for qualitative and quantitative changes in these constituents (Hobbie, 1994; Santoro et al., 2013). As an example, the foliar application of TDZ in *Mentha piperita* and *Salvia officinalis*, increased the content of the essential oil by 16%, besides resulting in increased levels of β- caryophyllene, α-humulene and other volatiles (Scravoni et al., 2006). *Achillea millefolium* L. is an important medicinal plant, distributed throughout the world, and is traditionally used to treat dyspepsia, inflammation, fever, cramps, as analgesic and anti-oxidant (Apelquist and Moerman 2011). Extracts of *A. millefolium* have shown effective in combating the virus that causes Newcastle disease in broilers (Rezatofighi et al., 2014). Another investigation, the plant has great potential for phytoremediation of soils contaminated with oil, with an increase in their antioxidant potential (Masu et al., 2014).

The yarrow has constituents of biochemical and pharmacological interest, as the flavonoids apigenin, luteolin, rutin and casticin (Pedneault el al., 2014). Another important metabolite produced by the plant is the essential oil, present in its leaves and inflorescences. The essential oil of yarrow is mainly composed of monoterpenes (30-80%), sesquiterpenes (8-62%) and of other compounds (1-3%), with over 140 terpene compounds identified in the essential oil (Kindlovits

Table 1. Variables analyzed of *in vitro* multiplication of *A. millefolium* under the influence of different concentrations of Thidiazuron, at 45 days of cultivation.

Thidiazuron (mg L^{-1})	Number of shoots	Shoot length (cm)	Dry mass of shoots (mg $shoot^{-1}$)
0.00	1.00[c*]	5.06[a]	0.21[a]
0.25	3.31[b]	1.34[b]	0.24[a]
0.50	2.60[b]	1.23[b]	0.20[a]
0.75	3.90[ab]	1.35[b]	0.17[a]
1.00	5.56[a]	1.67[b]	0.09[b]

*Means followed by the same letter in column do not differ by the Tukey's test ($p \leq 0.05$)

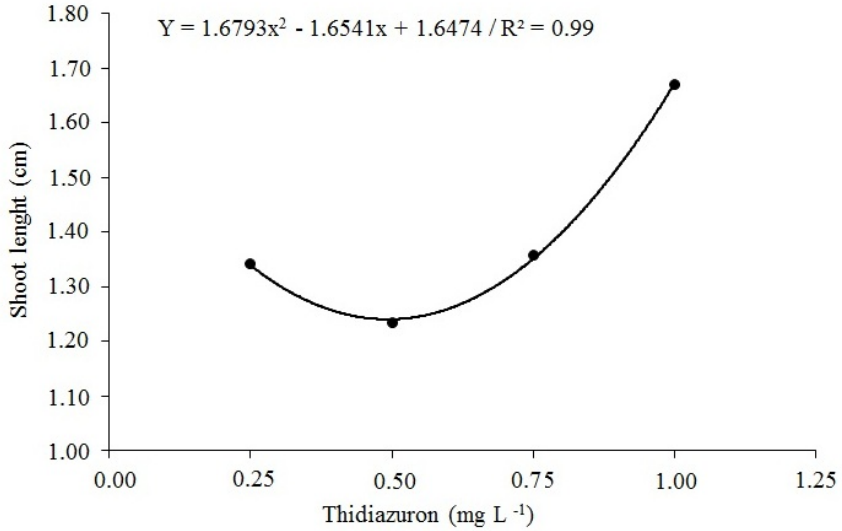

Fig 1. Linear regression of shoot length of *A millefolium* L. cultivated in MS medium with concentrations of Thidiazuron at 45 days. The shoot length is positively affected by increasing TDZ in the culture medium.

and Németh 2012). Given the above, studies on plant growth regulators are necessary to evaluate not only proliferation, but also to identify relevant changes in the profile of constituents, particularly in plants which produce essential oils, such as yarrow. Thus, the objective of this study was to evaluate different concentrations of Thidiazuron on *in vitro* multiplication of *Achillea millefolium* L. and its influence on the volatile fraction of leaves.

Results and Discussion

Shoot production

The yarrow shows positive response to application of TDZ in culture medium, (Table1). Regarding number of shoots, the concentration of 1.00 mg L^{-1} of TDZ It was the one with the higher of the treatments, averaging 5.56 shoots per explant inoculated. The variable length of shoots, there was an increase in length with increasing concentrations the regulator (Fig 1); however, there was no difference between treatments (Table 1). Similar results were observed in multiplication of some medicinal plants, as in *Pfaffia glomerata* (Flores et al., 2009); *Varronia curassavica* (Santos et al., 2013), and *Stevia rebaudiana*, (Lata et al., 2013) resulting in a high number of shoots and shoot length, being more effective than 6-benzilaminopurine. This response is due to the fact that Thidiazuron causes the breaking of the apical dominance, and thus increases multiplication areas and cell elongation, through physiological changes somewhat elucidated (Guo et al., 2013). It has been reported that TDZ hinders the action of cytokinin-oxidase, and this allows the proliferation of meristematic zones in the explants (Hare et al., 1994). The dry mass of shoots decreased proportionally with the increase in the regulator in medium of culture (Fig 2). Despite the higher number of shoots, treatment of 1.00 had the lowest amount of dry mass between treatments (Table 1). This response may be associated with source-sink relations, regulated by meristematic activity of shoots (Roitsch and Ehneb, 2000). As the *in vitro* resources, that nutrients and gas exchanges, are limited, produced assimilates are distributed between the shoots, which reduces their mass accumulation (Su et al., 2011). The Fig 3 shows the overall aspect of plants. Plants that were not under the influence of the regulator, showed normal development, no abnormalities or disorders, and the well-developed root system. In buds, leaves with hyperidricity and less formation of callus were observed. A study conducted by Turker et al. (2009) evaluated the micropropagation of *A. millefolium* through different plant growth regulators, and the authors concluded that TDZ is a potential plant growth regulator for this species, combining with giberellic acid (GA_3) and indolacetic acid (IAA). In both works, the responses indicate that TDZ can be used successfully for *in vitro* multiplication of yarrow and, in this study, the concentration of 0.75 mg L^{-1} shows better combination of number, length and dry mass of shoots.

Volatile compounds

The identification of volatile compounds was higher than 98%, where it is possible to observe that the number of compounds was higher (32) in the initial plantlets (Table 2). Younger plants are more subject to attack by pests, diseases and stressful environmental conditions, and the major number of volatile constituents being a form of protection of the plant (Croteau et al., 2000).

Comparing the initial plantlets (30 days) with the final plantlets (75 days), it can be observed that the age of plants

Table 2. Relative areas of volatile constituents of the leaves of *A. millefolium* L. cultivated *in vitro* under different concentrations of Thiadizuron.

Compounds	IR[1]	IP[2]	FP[3]	Thidiazuron (mg L^{-1})				
				0.0	0.25	0.50	0.75	1.00
Tricyclene	923	—	—	0.64	—	—	—	—
α-Thujene	925	0.96^{ns}	1.21	1.31	1.41	1.21	1.19	1.32
α-Pinene	932	1.43^{ns}	1.60	1.91	1.98	1.73	1.61	1.93
Camphene	947	0.85^{ns}	0.96	1.27	1.04	1.05	0.94	1.03
Sabinene	973	25.97^{d*}	37.29^{a}	36.30^{a}	34.78^{b}	31.09^{c}	31.49^{c}	32.93^{b}
β-Pinene	976	2.21^{ns}	2.32	2.44	1.99	1.76	2.11	1.92
6-metil-5-hepten-2-one	986	0.24^{bc}	0.23^{bc}	0.18^{c}	0.41^{ab}	0.49^{a}	0.27^{bc}	0.51^{a}
Myrcene	990	1.47^{b}	2.89^{a}	3.23^{a}	1.14^{b}	1.01^{b}	1.49^{b}	1.15^{b}
α-Felandrene	1005	—	—	0.20	—	—	—	—
α-Terpinene	1016	0.28^{ns}	0.37	0.98	—	0.25	0.24	0.24
para-Cymene	1023	0.82^{ns}	1.49	—	0.45	0.45	0.69	0.49
D-Limonene	1027	3.32^{ab}	5.28^{a}	5.49^{a}	2.57^{b}	2.26^{b}	2.88^{b}	2.32^{b}
1.8-Cineole	1030	8.47^{a}	10.53^{a}	12.51^{a}	5.11^{b}	4.76^{b}	5.73^{b}	5.74^{b}
1-hidroxi-nonene	1040	—	0.10	—	—	—	—	—
γ-Terpinene	1057	1.71^{a}	2.29^{a}	2.42^{a}	0.68^{b}	0.62^{b}	1.79b	0.70^{b}
trans-Sabinene hydrate	1065	2.88^{a}	2.38^{ab}	—	1.56^{bc}	1.39^{bc}	1.81^{bc}	1.25^{c}
Terpinolene	1088	0.46^{a}	0.51^{a}	0.51^{a}	—	—	0.33^{ab}	—
Thujanol	1098	1.56^{ns}	1.29	1.20	1.37	1.30	1.44	1.21
Borneol	1164	11.52^{a}	7.41^{b}	8.29^{b}	10.79^{a}	11.05^{a}	11.65^{a}	10.62^{a}
Terpinen-4-ol	1176	0.73^{ns}	0.68	0.63	0.81	0.75	0.73	0.70
α-Terpineol	1190	3.33^{ns}	2.01	2.26	2.77	2.18	2.81	2.96
Bornyl-acetate	1285	0.72^{ns}	0.47	0.54	0.46	0.64	0.73	0.65
α-Cubebene	1374	0.22^{a}	0.12^{b}	0.10^{b}	—	—	—	0.27^{a}
β-Elemene	1391	0.15^{b}	0.12^{b}	0.11^{b}	0.33^{a}	—	0.29^{a}	0.31^{a}
β-Cryophyllene	1417	12.12^{b}	8.45^{b}	7.521^{b}	17.26^{a}	18.26^{a}	16.02^{a}	17.89^{a}
Biciclofelandrene	1427	0.16	—	—	—	—	—	—
α-humulene	1451	1.40^{ab}	0.87^{b}	0.84^{b}	2.23^{a}	2.49^{a}	2.03^{ab}	2.35^{a}
E-β-Farnesene	1457	0.62^{b}	0.24^{b}	0.17^{b}	1.30^{ab}	2.05^{a}	0.82^{ab}	1.23^{ab}
γ-Muurolene	1475	0.24	—	—	—	—	—	—
β-Cubebene	1479	11.43^{a}	6.95^{ab}	4.20^{b}	6.07^{ab}	7.46^{ab}	7.66^{ab}	6.88^{ab}
α-Bergamotene	1495	0.65^{ns}	0.20	0.10	0.39	0.49	0.39	0.67
α-Farnesene	1509	2.79^{ns}	0.59	0.53	1.90	3.18	1.81	2.53
γ-Cadinene	1512	0.15	—	—	—	—	—	—
β-Sesquiphelandrene	1523	0.41^{ns}	0.21	—	0.25	1.07	0.50	0.55
Caryophyllene oxide	1580	0.21^{c}	0.14^{c}	0.13^{c}	0.41^{b}	0.47^{b}	0.28^{c}	0.58^{a}
Monoterpenes (%)		66.45^{b}	79.48^{a}	83.23^{a}	65.98^{b}	61.34^{b}	65.99^{b}	63.58^{b}
Sesquiterpenes (%)		30.64^{a}	17.92^{b}	13.71^{b}	30.20^{a}	35.50^{a}	29.84^{a}	33.30^{a}
Others (%)		2.54	2.03	1.94	3.40	3.34	3.99	3.12
Total identification (%)		99.63	99.42	98.58	99.58	100	99.83	100
Number of compounds		32	30	29	26	25	28	28

*Means followed for the same letters in line do not differ by Turkey's test ($p \leq 0.05$); ^{ns} Not significant; [1]Relative retention index n-alkane series (C_8-C_{18}) HP-5MS column in order of elution. Data are means of four replications of each treatment. — Undentified. [2]Initial plantlets (30 days in MS free-hormones); [3]Final plantlets (75 days in MS free-hormones).

influenced on the concentrations of the major constituents, as can be noticed with borneol and β-cubebene, showing higher values in initial plants. Many genes of secondary metabolism are regulated by cell maturation, thus, the tissue age promotes changes in the constituents (Mossab et al., 2015; Roberts et al., 2007). The terpenes influenced by the application of the regulator were: sabinene, 6-metil-5-hepten-2-one, myrcene, D-limonene, 1.8 cineole, γ-terpinene, *trans*-sabinene hydrate, terpinolene, borneol, α-cubebene, β-elemene, β-caryophyllene, E-β-farnesene, β-cubebene, and caryophyllene oxide (Table 2). Singh et al. (2008) cite that cytokinins affect metabolism of terpene synthases enzymes, having a direct effect of terpenes. The five major compounds, which were sabinene, 1.8-cineole, borneol, β-caryophyllene, β-cubebene, corresponding to approximately 68% of total constituents, which is consistent with other studies of characterization of volatile constituents of the species. Usually the medicinal properties of plants are assigned a few compounds, but in the yarrow are observed greater complexity of constituents with biological activity (Kindlovits and Németh 2012). The best contents of sabinene (37.29%), myrcene (3.23%), D–limonene (5.49%), 1.8 cineole (12.51%), γ-terpinene (2.42%) and *trans*-sabinene hydrate (2.88%) were observed in absence of regulator, thus not influenced by the action of TDZ. Already, the β-caryophyllene has the highest contents in the presence of TDZ, with contents between 16.02% and 18.26%. This result is justified by the observation made by El Keltawi and Croteau (1987), reported that increasing of activity of two enzymes terpene synthases, measured in sage and mint, with exogenous application of cytokinins. For yarrow, a significant increase in levels of β-caryophyllene when TDZ was present, indicating that this regulator favors the synthesis of this constituent. The cytokinins operate in different physiological and biochemical processes and gene expression in plants, thus, exogenous application of such substance can increase the production of compounds of

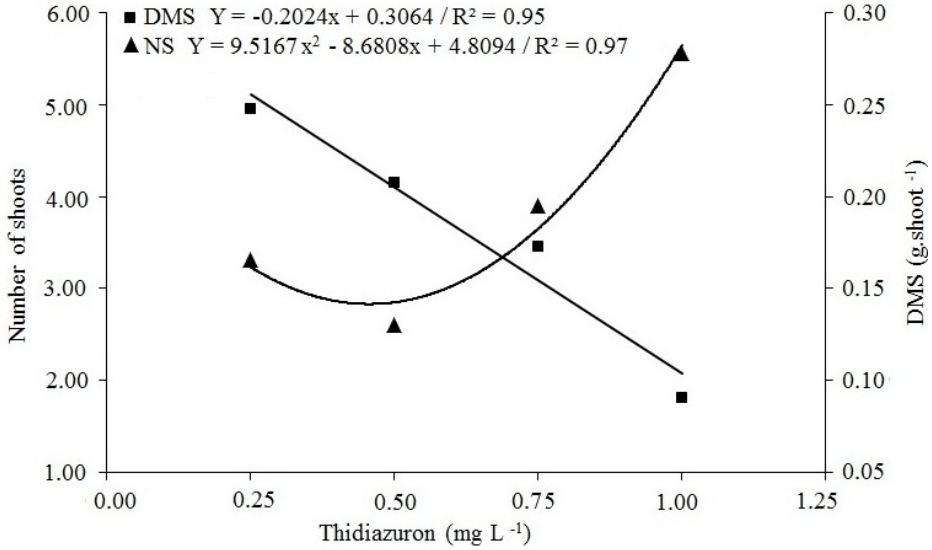

Fig 2. Linear regression of number and dry mass of shoots of *A. millefolium* L. cultivated in MS medium with concentrations of Thidiazuron at 45 days. The regulator increases the number of shoots, but decreases the dry mass per shoot. NS-number of shoots; DMS-dry mass of shoots.

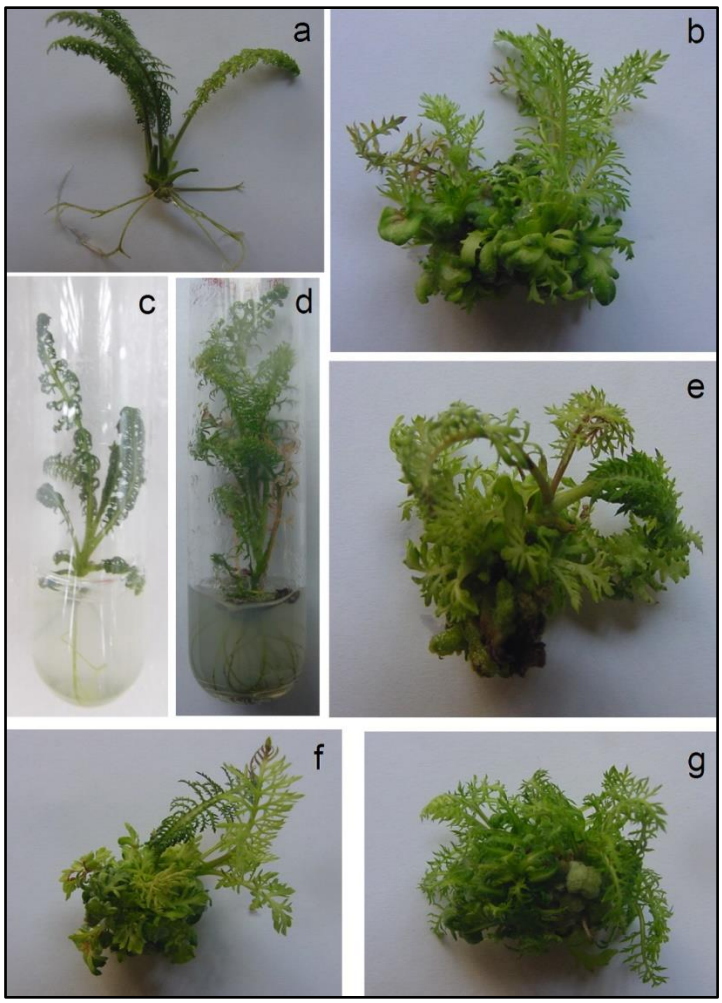

Fig 3. Overall aspect of *A.millefolium* L. cultivated *in vitro* **a** Control: free-hormone MS. **b** MS medium + 0.25 mg L^{-1} of TDZ. **c** Initial plantlets (30 days in free-hormone MS medium). **d** Final plantlets (75 days in free-hormones-MS medium). **e** MS medium + 0.50 mg L^{-1} of TDZ. **f** MS medium + 0.75 mg L^{-1} of TDZ. **g** MS medium + 1.00 mg L^{-1} of TDZ.

interest (Scravoni et al., 2006). Examples of the positive effects of cytokinin action of the application of kinetin and BAP resulted in increases in the levels of essential oil basil (Hazzoumi et al., 2014), lavander (Sudriá et al., 1999) and palmarosa (Khan et al., 2015), that indicate the feasibility of using these substances as for eliciting the production of essential oils. Another important aspect is related to classes of constituents. When confronted the initial plants and treatments with the regulator, the total concentration of monoterpenes and sesquiterpenes were similar. In regulator presence, there is an increased meristematic activity, as well as in plants "young", which reflects on the constituents classes. The observed result, the presence of the regulator in the culture medium decreases the monoterpenes content and consequently, increases the levels of sesquiterpenes. To conclude, this study is one significant step for research on terpenes of yarrow, being the first report which evaluated the influence of a plant growth regulator on volatile constituents *in vitro*.

Materials and Methods

Plant material

To initial establish *in vitro*, the terminal segment of rhizome was used. Plants were grown in a greenhouse under a fertigation system, on the washed sand. The nutrient solution was formulated with the following sources and concentrations (mg L^{-1}): $CaNO_3$:760; NH_4SO_4: 225; KCl 400; $MgSO_4$:375; Tenso Ferro®: 44; Mn SO_4: 6.72; HBO_3: 3.28; $ZnSO_3$: 0.56; $CuSO_4$: 0.36; $NaMo_4$:0.05 and monoammonium phosphate (MAP):110. Initially, explants were removed from the sand bed, reduced to 15 mm, washed with a few drops of liquid soap and rinsed with running water for 30 minutes. After rinsing, they were immersed in 70% alcohol for 10 seconds and placed in 50% sodium hypochlorite solution to stir for 20 minutes. In an aseptic laminar flow cabinet, explants were washed four times with autoclaved water, with the first two washings performed at 30 seconds, and two subsequent for 1 minute. Subsequently, they were excised to a size of 10 mm and inoculated into tubes containing 15 mL MS medium (Murashige and Skoog, 1962) with 30 g L^{-1} sucrose, 0.6% agar and pH adjusted to 5.7±1. After inoculation, the explants were kept in a growth chamber with cool white fluorescent lamps with 32 µmols $m^{-2}s^{-1}$ intensity, a 16-h photoperiod, at 25 ºC. There was a serious trouble with bacterial contamination, identified in 2-3 days after the onset (data not shown) and, in order to obtain a sufficient number of plants, two subcultures were performed. With the objective to do this, the plantlets established, without contaminants, for 30 days, without leaves and roots, were placed in flasks containing 30 mL MS + 0.50 mg L^{-1} TDZ. After 45 days, the shoots were isolated and inoculated into tubes with 15 mL MS and kept under the same environmental conditions. The multiplied plantlets, for 30 days, without TDZ, were cut to 10 mm length, washed twice with sterile water and inoculated into flasks, containing 40 mL MS medium with TDZ. The concentrations tested were: 0.25; 0.50; 0.75; 1.00 mg L^{-1} and the control without regulator. The design was completely randomized with five replicates per treatment and 5 flasks per replicate. At 45 days, the numbers of shoots was evaluated, as well as shoot length and dry weight of shoots (mg $plantlet^{-1}$). Thus, the aerial part of buds was dried in a forced air oven, with constant temperature of 45° C.

Analyses of volatiles

In order to assess possible changes in the volatile compounds arising from the use of the regulator, the analysis of the volatile fraction was performed in yarrow leaves. For comparison of the volatiles, samples were removed from the plants after 30 days on MS free-hormone medium, identified as "initial plantlet" (IP) and plants with 75 days were called "final plantlets" (FP). Four samples for each treatment (20 mg leaves – dry mass) were collected for the determination of volatile constituents. The analysis was performed on a system of gas chromatography Agilent® 7890A coupled to a mass selective detector Agilent® 5975C MSD (Agilent Technologies, CA, USA), operated by electron impact ionization at 70 eV in scan mode, speed of 1.0 scan s^{-1}, with an interval of acquiring masses of 40-400 m/z. A fused silica capillary column HP-5MS (30 m length x 0.25 mm internal diameter x 0.25 mm thick film) (CA, USA) was used.

Helium was used as the carrier gas, with a flow of 1.0 mL min^{-1}; injector temperatures, as well as the transfer line to MS, were kept at 230º C. The initial oven temperature was 60° C, followed by a temperature ramp of 3° C min^{-1} to 200 °C followed by a ramp of 10° C min^{-1} to 270 °C. The concentrations of the volatile fraction were expressed as a percentage of relative area of the chromatographic peaks. Retention indexes were calculated using the equation of Van den Dool and Kratz (1963). Constituents were identified by comparing their contents with the contents of a standard solution of *n*-alkanes (C_8-C_{18}), (Sigma-Aldrich ® USA) and by comparing the mass spectra of NIST (NIST, 2008) and with the literature (Adams, 2007).

The variables of multiplication analyzed were number of shoots, shoot length and dry weight of shoots. The data were subjected to analysis of variance and linear regression and the identificated compounds, had their means compared by the Tukey's test at $p \leq 0.05$ by the SAEG program (SAEG, 2007).

Conclusions

Due to medicinal importance of yarrow and its worldwide distribution, this work is an important contribution to the study of multiplication and production of *in vitro* constituents. The use of 0.75 mg L^{-1} of TDZ on MS medium was presented the best combination of variables: length, number and dry weight of shoots. In the regulator's presence, the classes of predominant constituents (mono- and sesquiterpenes) had levels near the young plants. In addition, its work serves as a basis for future studies, which aim to deepen understanding of the interaction between cytokinins and terpenes.

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