Elicitation of 7-methyljuglone in *Drosera capensis*

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Abstract

*Drosera capensis* L. (Droseraceae) is an important source of pharmacologically active 1,4-naphthoquinones including 7-methyljuglone which has been shown to have significant antimicrobial and antifungal as well as antituberculosis activity. In this study, we report on the production of 7-methyljuglone in *D. c* apensis under *in vivo* conditions by salicylic acid and jasmonic acid elicitation and by applying different strengths of media as well as different levels of total inorganic nitrogen (N) and ratios of nitrate (NO3−) to ammonium (NH4+) to a plant tissue culture medium. The amount of 7-methyljuglone produced was highest at 60 mM total nitrogen and at a 50:50 nitrate to ammonium ratio, which is ten-times higher when compared to the normal basal Murashige and Skoog growth medium. Elicitation of 7-methyljuglone in greenhouse grown plants using salicylic acid and jasmonic acid showed that the amount of 7-methyljuglone was the highest in the shoots of plants elicited with 50 µM of salicylic acid and jasmonic acid after 48 h and 3 h respectively. In roots, the highest amount of 7-methyljuglone was found in plants treated with 50 µM of salicylic acid and 100 µM of jasmonic acid after 1.5 h. The 7-methyljuglone concentration is generally higher in the roots than in shoots.

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1. Introduction

The most widespread genus of carnivorous plants is *Drosera* (Sundew) with about 130 species found worldwide (Didry et al., 1998; Bekesiova et al., 1999; Hook, 2001; Kawiak et al., 2003). *Drosera* plants grow mostly on acidic soils that are often deficient in nitrogen and phosphorous (Chandler and Anderson, 1976; Stewart and Nielsen, 1992; Adamec, 1997). They are rich in naphthoquinones (Hirsikorpi et al., 2002) and also contain glucosides of quinones (Budzianowski, 2000; Hirsikorpi et al., 2002; Panichayupakaranant and Tewtrakul, 2002; Kämäräinen et al., 2003) of which some are used as antispasmodic agents in the treatment of respiratory tract ailments (Didry et al., 1998; Hook, 2001; Kawiak et al., 2003). The naphthoquinone concentration in plants, however, is dependent on the growth season and differs between plant parts (Bonnet et al., 1984; Hook et al., 1997; Kämäräinen et al., 2003).

The major naphthoquinones found in the *Drosera* genus are plumbagin (2-methyl-5-hydroxy-1, 4-naphthoquinone) and 7-methyljuglone (7-MJ, 7-methyl-5-hydroxy-1, 4-naphthoquinone) (Hirsikorpi et al., 2002). 7-Methyljuglone has shown to be inhibitory to several insects and highly toxic to fungal pathogens (Seigler, 1998). The antifungal activity of 7-MJ isolated from *Euclidean* has already been reported (Watt and Breyer-Brandwijk, 1962; Steffen and Peschel, 1975). 7-MJ also has significant antituberculosis activity (Lall et al., 2005; Bapela et al., 2006; Van der Kooy et al., 2006) and the compound is a potent inhibitor of 12-hydroxyicosatetraenoic acid, which is a critical signalling molecule in tumour metastasis (Tang and Honn, 1994) and atherosclerotic processes (Nakao et al., 1982).

7-MJ production has been investigated in cell suspensions of *Drosera capensis* (Fig. 1) (Hook et al., 1997; Hook, 2001). Cell suspensions grown in bioreactors, can be an effective system to produce bioactive compounds (Yanpasian et al., 1999; Walker et al., 2002). More over *in vitro* condition is also a good opportunity for modification of environmental conditions, including medium ingredients, to obtain the optimal condition.
However, there is no information available on elicitation studies or optimization of tissue or cell suspension culture system for targeting of the 7-MJ enhancement in D. capensis.

We have therefore investigated in this study the elicitation of 7-MJ production in D. capensis under in vitro and modified in vitro conditions to increase 7-MJ production in D. capensis. For that purpose, the effect of different factors was studied on 7-MJ production including, elicitation of 7-MJ production by salicylic acid (SA) and jasmonic acid (JA) in in vitro experiments. The application of different nitrogen concentrations in combination with ratios of nitrate (NO$_3^-$) to ammonium (NH$_4^+$) in in vitro conditions was also studied in vitro.

2. Materials and methods

2.1. Plant material

Greenhouse grown plants and seeds of D. capensis were collected from the Botanical Garden of the Plant Science Department, University of Pretoria. Seeds were stored at 4 °C prior to use.

2.2. D. capensis seed germination

For the determination of the 7-MJ concentration in in vitro grown plants, seeds were germinated after surface-sterilization (70% ethanol for 30 s, 0.5% sodium hypochlorite for 30 min) and rinsing (3 times with sterile distilled water). Seeds were germinated on water agar medium containing 0.5% sucrose as the only ingredient and agar (0.7% w/v). The medium was adjusted to pH 5.7 before autoclaving for 20 min at 1.05 kg/cm$^2$ and 121 °C. Seeds were then incubated for germination at 25 °C under a 16/8 h photoperiod with light intensity of 30 µE m$^{-2}$ s$^{-1}$. Twenty five seeds were germinated in each Petri dish.

2.3. In vitro nitrogen treatments and media modification

10-day old seedlings from the germination study were used in this experiment. All media were supplemented with MS vitamins. The pH of the media was adjusted to 5.7 prior to autoclaving for 20 min at 1.05 kg/cm$^2$ and 121 °C. The cultures were incubated at 25 °C under a 16/8 h photoperiod with light intensity of 30 µE m$^{-2}$ s$^{-1}$. Six plants were used for each treatment.

2.3.1. Medium modification

The seedlings were cultured on hormone-free 1/3, 1/2 and full-strength MS (Murashige and Skoog, 1962) and B$_5$ (Gamborg et al., 1968) media in culture vessels (Magenta No 7). The cultures were incubated as described before. After an eight week incubation period the plant samples were harvested, dried at 50 °C and extracted with dichloromethane and analyzed with HPLC to determine the concentration of the 7-MJ content.

2.3.2. Nitrogen content modification in MS medium

Since Drosera plants grow in poor nutrient-conditions and they gain their nutrients, especially nitrogen and phosphorous from captured insects (Kämäräinen et al., 2003), another experiment was performed to determine the response of Drosera plants to different quantities and sources of inorganic nitrogen. In this experiment the inorganic nitrogen content in combination with the ratio of ammonium to nitrate in MS basal medium was modified. Two inorganic nitrogen levels, 30 and 60 mM and four NO$_3^-$:NH$_4^+$ ratios (50:50, 67:33, 75:25 and 100:0) were used according to Niedz (1994). MS medium contains 60 mM of inorganic nitrogen at a ratio of NO$_3^-$:NH$_4^+$ (67:33) was used as control medium. The result medium modification experiment showed that the concentration of 7-MJ was very low after 8 weeks and we therefore decided to harvest the plants in this experiment after 3 months.

2.4. In vivo elicitor treatment

Greenhouse grown plants were used for the elicitation of 7-MJ experiment in vivo. 3-month old Drosera plants were grown in pots filled with peat and washed sand (2:1) and were irrigated with distilled water only. SA and JA were applied as elicitors separately at different concentrations (0, 20, 50, 100 µM) on greenhouse grown plants. A stock solution of SA was prepared in deionised water. JA was first dissolved in ethanol and then diluted with deionised water (Biondi et al., 2000). For elicitor treatment, shoot tips of plants were excised with a scalpel and 2 µl of elicitor solution was placed on the excised surface of the shoot tips. Three individual plants were used for each treatment. Control plants were treated with distilled water in a similar procedure to the treated plants. The shoots and roots of treated and control plants were harvested separately at 1.5, 3.0, 6.0, 12.0, 24.0 and 48.0 h after treatment, dried in oven at 50 °C and weighed. The concentration of 7-MJ was measured three times by HPLC analysis.

2.5. Measurement of 7-MJ concentration

At the time of harvest the plant material of all replicates was pooled together according to Kirakosyan et al. (2004). Dichloromethane extracts of pooled samples were air-dried and dissolved in acetonitrile to a concentration of 10 mg/ml. Samples (10 µl) were subjected to an isocratic elution on a reverse phase HPLC system (5 µ, C$_{18}$ column, 150×4.6 mm).
The chromatographic system consisted of a 9012 pump (Varian, USA) connected to a manual sample injector 7161 (Rheodyne, USA) (Walker et al., 2002). The absorbance at 430 nm was measured by a UV6000LP photodiode array variable UV/VIS detector (tPS, USA). The mobile phase consisted of 62.5% acetonitrile, 32.5% water, and 5.0% aqueous citric acid and the flow rate was 1 ml/min. 7-MJ was quantified at 430 nm by comparison with a defined standard (Van der Kooy and Meyer, 2006) based on the peak area. PC 1000 software was used for peak integration analysis.

### 2.6. Statistical analysis

Analysis of variance (ANOVA) was used to determine the significance of variance. Tukey’s significant difference test was applied to determine the groups according to Compton (1994). Mean differences with a $p$ value greater than 5% were regarded as non-significant.

### 3. Results and discussion

The production of 7-MJ in *D. capensis* plants under *in vitro* conditions was influenced by MS medium strengths after eight weeks of incubation. The lowest 7-MJ production in the *in vitro* grown plants was found in the plants that were grown on 1/3-strength MS medium ($p<0.01$). No significant difference in the 7-MJ concentration was found when plants were grown on full-strength MS, 1/2 MS or B5 medium (Fig. 2). Although the basal MS medium has previously been reported as a medium for the tissue culture of the *Drosera* genus (Crouch et al., 1990), the results of this study showed that full and 1/2 strength MS and full strength of B5 medium are also suitable for the 7-MJ production in *D. capensis* under *in vitro* conditions. *Drosera* species usually grow in nutrient-poor habitats, low in nitrogen and phosphorous. Insects are a rich source of nutrients, particularly in nitrogen (Juniper et al., 1989). It has also been reported that *Drosera* plants are not an obligate insectivore, but

![Fig. 2. Comparison of 7-MJ contents in intact plants of *Drosera capensis* grown in different media *in vitro* with those in green house plants (control). Values of the bars with different letters are significantly different ($p<0.01$) according to ANOVA test ($n=6$).](image2)

![Fig. 3. Effect of different levels of total nitrogen, 30 and 60 mM and ratios of nitrate to ammonium on 7-MJ concentration in intact grown *Drosera capensis* plants. The ratio of nitrate to ammonium in original MS basal medium is 67:33, which is considered as the control in this experiment. Values of the bars not followed by the same letter are significantly different ($p<0.01$) according to ANOVA test ($n=3$).](image3)
that insectivory may provide the plant with an alternative source of nutrients (Givnish et al., 1984). In another study D. binata and D. capensis were fertilised with nitrogen and phosphorous and it was observed that both species benefited from soil nutrient addition (Stewart and Nilsen, 1993).

The concentration of 7-MJ after three months was 0.07 g/kg dry weight when 60 mM total inorganic nitrogen and NO$_3$-$:$NH$_4$$_2$ at a ratio of 50:50 was used (Fig. 3). This is more than ten-fold higher when compared to basal full-strength MS medium (control plants) containing 60 mM total inorganic nitrogen with a NO$_3$:$$NH_4$$_2$ ratio of 67:33, where the concentration of 7-MJ was found to be 0.006 g/kg dry weight. In contrast, among the treatments with 30 mM total inorganic nitrogen, 7-MJ content was found highest in plants treated with a ratio of 100:0, NO$_3$:$NH_4$$_2$. There is evidence of a link between the forms of N uptake and N assimilation (NO$_3$ and NH$_4$$_2$) and the formation of intercellular H$^+$ and OH$^-$ (Kirkby and Knight, 1977; Van Beusichem et al., 1988). Inorganic N uptake can strongly influence the pH of the medium since the uptake of NO$_3$ and NH$_4$$_2$ changes the pH in the medium in opposite directions (Niedz, 1994). The absorption of the NO$_3$ anion results in an increase in pH to maintain charge neutrality, generally by the extraction of HCO$_3$. Conversely, the absorption of NH$_4$ cations results in the production of H$^+$ which is excreted into the medium. This lowers the pH and reduces any further cation uptake by competitive effects (Kirkby and Mengel, 1967). Ammonium in the medium can be consumed faster than nitrate, and during this time the pH in the medium decreases (De Block, 1990; Fracago and Echeverrigaray, 2001). In sweet orange callus, 93% of the variation in pH of the medium was dependent on NO$_3$:$NH_4$$_2$ ratios (Niedz, 1994). The role of pH as a necessary factor in the synthesis and composition of naphthoquinones has been demonstrated (Kurobane et al., 1980; Medentsev and Akimenko, 1992).

Fig. 4. Effect of different concentrations of salicylic acid on 7-MJ synthesis in Drosera capensis shoots and roots at different intervals. Values of the bars not followed by the same letter are significantly different ($p<0.05$) according to the ANOVA test ($n=3$).
1998). Obtaining the highest amount of 7-MJ in this study under the 60 mM total inorganic nitrogen and NO$_3^-$:NH$_4^+$ at a ratio of 50:50 condition can be attributed to the decreased pH. In another study the plumbagin content in *Plumbago rosea* was increased by using double the amount of (NH$_4$)$_2$SO$_4$ in B$_5$ medium (Panichayupakaranant and Tewtrakul, 2002). In fungi of the genus *Fusarium* the biosynthesis of naphthoquinone pigments occurred at pH 2.8–4.0, by using (NH$_4$)$_2$SO$_4$ in the medium as nitrogen source (Medentsev et al., 2005).

The concentration of 7-MJ in the shoots of *D. capensis* plants, which were elicited with SA showed that after 48 h of treatment of 50 µM SA, shoots produced the highest level of 7-MJ (0.013 g/kg dry weight). This was 2.1-times higher than the 7-MJ contents in the shoots of untreated control plants at the same time (0.006 g/kg dry weight) (Fig. 4-shoot). The highest amount of 7-MJ in the roots was observed in plants elicited with 50 µM SA after 1.5 h treatment (0.028 g/kg dry weight). The 7-MJ concentration in this sample was 2.3-fold higher when compared to the 7-MJ contents in the roots of control plants with 0.012 g/kg per dry weight after 1.5 h treatment (Fig. 4-root). Overall, we found that SA was effective in the enhancement of 7-MJ production in both roots and shoots, but it is more effective in eliciting 7-MJ production in the roots. In contrast to our results, it has been reported earlier

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**Fig. 5.** Effect of different concentrations of jasmonic acid on 7-MJ synthesis in *Drosera capensis* shoots and roots. Values of the bars not followed by the same letter are significantly different (p<0.05) according to the ANOVA test (n=3).
that in cell cultures of *Hypericum perforatum*, the application of SA had no effect on the concentration of another naphthoquinone, hypericin (Walker et al., 2002).

The amount of 7-MJ was the highest (Fig. 5-shoot) in shoots of plants elicited with 50 μM of JA after 3 h of treatment (0.017 g/kg dry weight). This was 2.8-fold higher than the 7-MJ content after 3 h of treatment in the shoots of untreated control plants. Under these conditions, the highest amount of 7-MJ was synthesized in the roots (0.028 g/kg dry weight) after 1.5 h of treatment, which was 2.3-times higher than the 7-MJ in the roots of control plants at the same time (Fig. 5-root). In plants elicited with different concentrations of JA, the content of 7-MJ in the roots reduced significantly (p < 0.05) between 1.5 and 6 h of treatment and after that, the content of 7-MJ stayed relatively constant until 48 h, except for a significant increase at 24 h (p < 0.05). The elicitor, JA and its derivates MeJA are considered to be involved in a part of the signal transduction pathway that activates particular enzymes catalyzing biochemical reactions to form defence compounds of low molecular weight in plants, such as polyphenoles, alkaloids, quinones, terpenoids, and polypeptides (Mizukami et al., 1993; William et al., 1996; Ding et al., 2004). JA and MeJA do not appear to be specific to a particular class of secondary metabolites, since a wide spectrum of compounds is elicited after exposure to exogenous JA or MeJA (Gundlach et al., 1992). The results of this study therefore confirm previous results (Walker et al., 2002; Ding et al., 2004) where an enhancing effect of JA and its deriviate, MeJA on hypericin, a naphthoquinone, has been found in cell cultures of *H. perforatum* and also the red naphthoquinone compounds of cultured *Onosma paniculatum* cells.

Overall, the present study has demonstrated for the first time that the elicitation of 7-MJ by SA and JA at the concentrations used in this study is an effective way to increase the amount of 7-MJ in both roots and shoots of *D. capensis*. The results of this study also showed that the yield of 7-MJ in plants treated with SA and JA was higher than intact plants (no wounding, no elicitation) whereas the 7-MJ contents in the shoots and roots were 0.008 and 0.009 g/kg dry weight respectively (This sample was prepared with the same procedure to the other samples in this study). The increased 7-MJ can be attributed to the stressed condition in plants, which was derived by applying SA and JA on *D. capensis* plants. Further, accumulation of 7-MJ in the roots of *D. capensis* is generally higher than in shoots. However, there is a significant interaction between concentration of elicitor (SA or JA) and time of harvesting (p < 0.01) for the 7-MJ concentration in the shoots and roots of *D. capensis*. The results of this study also showed that the production of 7-MJ can be influenced by the nitrogen concentration and the ratio of nitrogen sources. Although we cannot directly compare our *in vitro* and *in vivo* results, treating *D. capensis* with inorganic nitrogen in combination with ratio of nitrogen sources (nitrate and ammonium) is an effective way to increase 7-MJ production. Using nitrogen would be less expensive and therefore a more cost-effective strategy for elicitation than using chemical elicitors like SA and JA in a commercial approach for the 7-MJ production. This experiment still has to be carried out *in vivo* to confirm the positive effect of nitrogen and its sources on 7-MJ synthesis in *D. capensis*.

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### References


