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Table: 1, Figs : 3

Cytotoxicity of tea tree oil in tobacco cells

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ABSTRACT

This study analyzed the effects of essential oil from *Melaleuca alternifolia* (tea tree oil) on tobacco Bright Yellow 2 cells to understand the cytological basis for its allelopathic properties. The minimum cidal concentration values of tea tree depended on duration of treatment. Cell viability was lost even at lower concentrations, if exposure to the essential oil was sufficiently long. Loss of vitality corresponded to rapid leakage of fluorescent dye 7-amino-4-chloromethylcoumarin from the vacuoles and cells. These cytological changes were monitored in real time and documented using time-lapse photography. The results have shown that tea tree oil has a major effect on membrane permeability not only in bacteria and fungi but also in plant cells. *Melaleuca* oils' mode of action at the cellular level can explain the allelopathic properties (inhibition of germination and seedling growth) observed in nature. It suggested the potential use of tea tree oil for weed control.

Keywords: Essential oil, *Melaleuca,* membrane permeability, tobacco cells, tonoplast, vacuole

INTRODUCTION

Higher plants synthesize several essential oils with important ecological roles (4,8). Monoterpenes, the main constituents of these substances, interfere() with basic biological processes [DNA replication (6), mitochondrial respiration (13), enzyme activities (18), seed germination (17) and plant growth] (1,6, 8,14,21). In general, cytotoxic properties of terpenes have been attributed to their ability to accumulate in membranes and thereby affecting their permeability (11,19). The terpene content in plants varies with plant spp. and season (8). The monoterpenes are selective in their activities (22) and the synergistic and antagonistic interactions are the properties of essential oils (20,22).

Leaf extract of *Melaleuca alternifolia* often called 'tea tree oil' (TTO), has been studied as antimicrobial agent (3), however, its allelopathic properties are not known. Essential oils from various other *Melaleuca* spp. inhibit seed germination and growth of radish, and the extent of inhibition depends on plant species and the source of extract (23). Our preliminary studies showed that TTO is potent inhibitor of seed germination (unpublished results). In this study, we aimed to analyze the effects of TTO on individual plant cells, to better understand the basis of its allelopathic properties. As a model system we used the tobacco Bright Yellow 2 (BY-2) cells grown as a fine callus suspension

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culture. Exponentially growing cells were exposed to various concentrations of TTO for different lengths of time, and minimum cidal concentrations were determined. Loss of cell viability coincided with the release of a fluorescent dye from cells, specifically for vacuoles. Time lapse microphotography was performed to monitor changes in tonoplast permeability in real time.

MATERIALS AND METHODS

To analyze the effects of TTO in plant cells, tobacco BY-2 fine callus suspension culture was used as a model system, because it consists of small clusters of cells, allowing relatively uniform exposure of cells to the chemical. Also, BY-2 cultures have been extensively characterized and are highly amenable to cytological analysis (16). Sensitivity of tobacco cells to TTO was determined by incubating exponentially growing cells with various concentrations of TTO for different durations of time. In controls, mineral oil replaced the TTO. After treatments, cells were washed and plated out on medium without TTO. After 3-weeks of incubation, plates were scored for callus growth.

Tobacco BY-2 cells (*Nicotiana tabaccum* L. cv. Bright Yellow 2) were maintained in Murashige and Skoog (MS) salt medium (Sigma-Aldrich) on a rotary shaker (125 rpm) at 27°C in dark. Every week, 10 mL of stationary phase cells was transferred into 90 mL fresh medium.

Treatment with Tea Tree Oil

The commercially available (100% pure pharmaceutical grade) terpinen-4-ol chemotype (3) essential oil from *Melaleuca alternifolia* (Tea Tree Therapy, Inc) with minimum of 36% (v/v) terpinen-4-ol and 3% (v/v) 1.8-cineole was used in the experiments. Cells obtained from 3- days old cultures, were resuspended in culture media containing 0.1% (v/v) Tween 80 (Sigma-Aldrich) and different amounts of TTO. In control, up to 1% (v/v) light Mineral Oil (Fisher Scientific) was used instead of TTO, in presence of 0.1% Tween 80. To determine the minimum cidal concentrations, cells were treated with different TTO concentrations for various lengths of time. Thereafter, cells were allowed to settle. Supernatant was removed and replaced with fresh medium. After gentle mixing, the washing procedure was repeated twice. Cell were plated on MS agar and incubated at 27°C in dark. Plates were visually monitored to detect cell growth for 3-weeks. Three parallel plates were used to observe and score cell growth for each TTO concentration. The experiment was carried out twice.

Cell Staining – Microscopy

For vital staining fluorescein diacetate (FDA, Sigma- Aldrich) was used at 5 µg/mL for 5 min. To detect dead cells, the DNA stain Sytox ® Orange (Molecular Probes) was applied to cells at 0.5 µg/mL for 20 min.

Vacuoles were visualized using 7-amino-4-chloromethyl coumarin (CMAC, Cell Tracker Blue CMAC-Molecular Probes) at 5 µg/mL for 25 min. Cells were observed with Olympus BX50 microscope with a BX-FLA reflected light fluorescent attachment. The vital staining procedures were performed twice at each TTO concentration and the experiment was repeated four times.

Fluorescence measurement

Blue fluorescence was quantified using Photoshop CS2 (Adobe Systems Inc.). Vacuoles in images were selected and blue channel mean intensities were calculated using the program's histogram tool. Means and standard deviation of samples and linear regression for 3 independent experiments were calculated from data collected using identical instrument settings and graphed with Sigmaplot 2001 (Systat Software Inc.).

Time-lapse photography

Staining with CMAC was done as above. Cells were washed thrice in MS medium to remove excess dye and were resuspended in culture medium [1% (v/v) TTO and 0.1% Tween 80]. Aliquots were immediately transferred onto a microscope slide and photographs were taken at 1 min intervals using a SPOT RT 18 SE camera (Diagnostic Instruments). To minimize photo-bleaching, low intensity illumination light was applied only for the duration of time that the photographic images were taken. The experiment was performed four times.

RESULTS AND DISCUSSION

Serial dilution of TTO in MS medium was used to determine the minimum cidal concentrarion in tobacco callus suspension culture (see Material and Methods). In Table 1, the plus (+) sign showed colony formation, while, the minus (-) sign marks no cell growth on plates. TTO had strong cytotoxic effect on BY-2 cells with clear dependence on TTO concentration and duration of treatment. While incubation of cells with mineral oil (control) or up to 0.04% (v/v) TTO for 24 h allowed callus growth, treatment with 0.065% (v/v) TTO was lethal to cells. An apparent threshold concentration was observed between 0.125 and 0.250 % (v/v) TTO, thereafter, a rapid loss of viability occurred in less than 20 min after treatment (Table 1).

Table 1. Effects of tea tree oil (TTO) concentrations and treatments duration on callus growth of tobacco BY-2 cells

TTO Conc.	Treatments duration (min)						
(%)	20	40	60	120	240	360	1440
0.00	+	+	+	+	+	+	+
0.02	+	+	+	+	+	+	+
0.04	+	+	+	+	+	+	+
0.065	+	+	+	+	+	+	-
0.125	+	+	+	+	+	-	-
0.25	-	-	-	-	-	-	-

Cells were washed and plated on MS medium containing no TTO. Plates were incubated for 3 weeks, and scored for observable callus growth (+) or no growth (-).

The concentration values at which TTO was lethal to tobacco BY-2 cells are in accordance with data from a study where monoterpenes (linalool, α-terpinene or β-pinene) were added separately to a *Pelargonium fragrans* suspension culture in exponential growth phase for 24 h (2). In our experiments, we also used logarithmically growing callus suspension cultures, but did not test the effect of TTO on other growth phases. We found

that TTO's minimum cidal concentration values can only be properly interpreted, if incubation times are considered (Table 1). The time of exposure, played a critical role in TTO's cytotoxicity. Even at lower concentrations, if exposure to TTO was sufficiently long, viability of cells was affected. For instance, whereas colony was formed 6-hours after exposure to 0.065% (v/v) TTO, viability was completely lost in cultures, where the same oil concentration was used for 24 h (Table1). These data were confirmed using fluorescence microscopy. Double staining of tobacco callus suspension with vital stain FDA and dead cell specific dye Sytox orange showed that while most cells survived the treatment with 0.04 % (v/v) TTO for 24 h, no live cells were found using 0.065% (v/v) (Fig.1). The presence of aggregating cell clusters and dense pattern of Sytox nucleic acid staining suggest that loss of vitally is associated with significant morphological and structural changes in tobacco suspension culture (Fig.1). The longest incubation time we tested was 72 h, where some cells in clusters began to display weaker nuclear and cytoplasmic FDA staining at as low as 0.04 % (v/v) TTO. No change in FDA staining was observed at 0.02 % (v/v) TTO or in the control (photographs not shown).

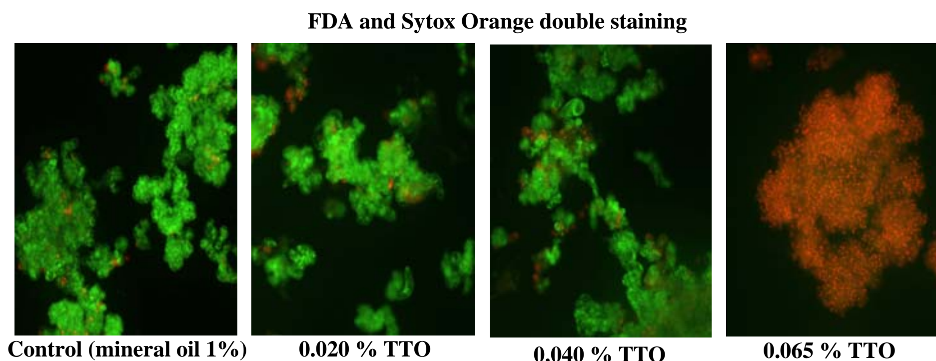

Figure 1. Tobacco BY-2 callus suspension cultures were exposed to various concentrations of TTO for 24 h, and then stained with FDA to detect live cells (green) as well as with Sytox orange, specific for dead cells (red). Green and red fluorescence images of the same samples were superimposed. The control contained 1% (v/v) mineral oil instead of TTO.

This time-concentration factor could play an important role in *Melaleuca* oils' inhibitory effects on germination and seedling growth. Terpenes can accumulate and remain in the soil for months (15), creating a toxic environment for plant development. In callus suspension cultures, dividing the plant cells were more sensitive to essential oils than in stationary phase (2). Therefore the dividing cells in the meristematic zones of seedlings may be more susceptible to damage from some essential oils. That is why root and hypocotyl growth are inhibited by certain terpenes (10,14). Using electron microscopy, Lorber and Muller observed that volatile inhibitors from *Salvia leucephylla* caused severe damage in cell organelles in the root tip of *Cucumis sativus,* including disrupted mitochondrial membranes, loss of identifiable Golgi apparatus and irregularly shaped plastids (9). We wanted to know, whether TTO causes similar damage in BY-2 cultures. We speculated that fluorescent dyes that specifically accumulate in cell

compartments could be used to monitor the cytological changes. Accelerated release of a dye from a cell organelle can be diagnostic to altered membrane permeability. Although little is known about how the tonoplast is affected by essential oils, due to their large size, vacuoles are convenient targets for cytological observations. Moreover, they play an essential role in several cellular processes, including the maintenance of homeostasis (12). Thus induced changes in tonoplast's permeability may suggest severe perturbations in vital cellular functions. We used Tracker Blue CMAC fluorescent dye, which is specifically and stably taken up by the vacuoles (Figure 2.). We have found that unlike FDA, fluorescence by CMAC was not interfered with even by high concentrations and prolonged co-incubation with TTO. After a short exposure to CMAC, TTO was applied at 0.065% (v/v). Samples were taken at regular intervals for 25 h and fluorescence intensities were determined in the vacuoles. Figure 2 shows that during the first 22 h of culture, fluorescence remained essentially unchanged.

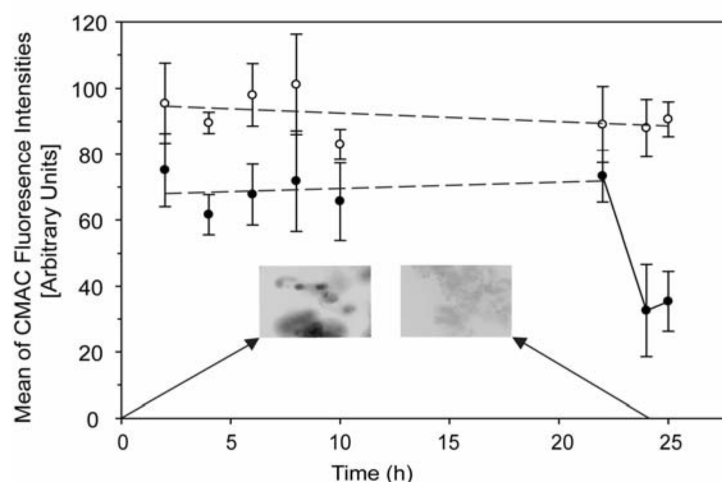

Figure 2. Tobacco BY-2 cells pulse-labeled with the vacuole specific fluorescence dye CMAC were incubated with 0.06% TTO for 25 h. Blue fluorescence intensities in vacuoles were calculated for samples taken at regular intervals. Sample mean values with standard deviations (n=3) and corresponding linear regression (dashed lines) are shown. Insets are photographs of cells after 0 and 24 h treatment with TTO.

There was a rapid decrease in emitted blue light intensities between 22 h and 24 h, perhaps owing to change in permeability of tonoplasts. As a result, CMAC leaked from the vacuoles (Fig. 2). In control, little change in fluorescence intensities occurred over 25 h. The time of CMAC release at 0.065 % (v/v) TTO seems to be similar to our results in Table 1, where loss of viability occurred less than 24 h after incubation, at the same TTO concentration. To better monitor in real time the effect of TTO on individual cells, a much shorter time frame for treatment was required. Hence, TTO concentration was increased to 1%. Preliminary studies had shown that at this concentration, cells die within minutes of

exposure to TTO (results not shown). After pulse-staining cells with CMAC, TTO was added and samples were immediately mounted on a microscope slide (Fig. 3).

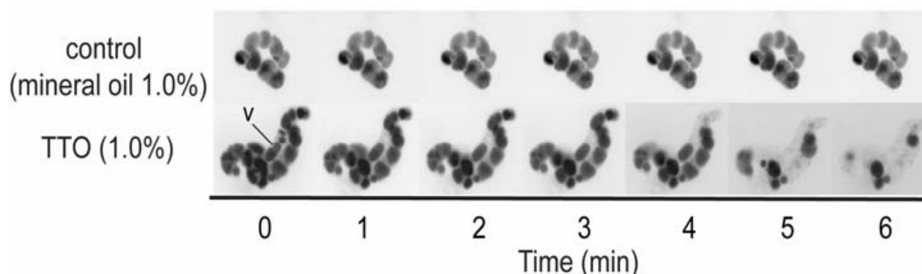

Figure 3. BY-2 cells pulse-labeled with CMAC were exposed to 1.00% (v/v) TTO or, as control, (1.00 % (v/v) Mineral Oil. Samples were immediately mounted on microscope slides and photographed at 1 min intervals at 200x magnification. To assist better visualization, pictures are photographically inverted. v: vacuole

CMAC specifically localized in the vacuoles was readily visible at 200x magnification. In some cells within the microcalli, coalescing vacuoles and release of dye was observable after 1 min of treatment (Fig. 3). However in majority of cells, a relatively sudden and simultaneous loss of fluorescence from the vacuoles was observed by 4 min. By 6 min, most of the dye had diffused out of the cells with a concomitant increase in background fluorescence (Fig. 3). These findings also suggest that changes in permeability of the plasmalemma may have preceded those of tonoplast. Control cells showed very little change in CMAC fluorescence 6 min after incubation (Fig. 3). The relatively rapid and simultaneous release of vacuolar CMAC at 1% (v/v) TTO was reminiscent to that observed when 0.065% TTO was used (Fig.2), even though it occurred on a different time scale (22-24 h).

Essential oils of *M. alternifolia* are known to disrupt the cellular membranes in *Candida*, *Saccharomyces* and other fungal genera (5). The data presented here clearly indicate a similar scenario in plant cells. The microscopic techniques used on non-fixed material have allowed us for the first time, to visually demonstrate in real time that TTO altered the membrane permeability in plant cells. We have shown that BY-2 microcalli maintained in liquid culture are irreversibly damaged even at low TTO concentrations, provided that the cells are exposed to the essential oil for long enough time. This study did not answer, as to which of the approximately 100 components of TTO has/have the strongest cytotoxic effects on tobacco cells. Although, based on studies in other organisms, terpinen-4-ol is a putative candidate (3), synergistic and antagonistic effects among terpene components may also play a role. Further experiments are needed to address this issue. The major cytological changes observed in BY-2 cells shown above may explain how the TTO inhibited the germination. A better understanding of the mode of action of essential oils or most effective combinations of their components holds out the possibility of developing highly efficient and yet environment friendly alternative weed control substances (10).

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