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SHORT-TERM RESPONSE OF SOIL MICROORGANISMS TO ESSENTIAL OILS WITH ALLELOPATHIC POTENTIAL EXTRACTED FROM MEDITERRANEAN PLANTS

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ABSTRACT

Essential oils (EOs) with allelopathic compounds have been used to reduce or avoid weed germination and growth. The aim of this study was to evaluate the potential phytotoxic effects of EOs extracted from different Mediterranean plants on soil microbial biomass and activity. EOs were extracted from leaves of *Eucalyptus camaldulensis* Dehnh (EUC); *Eriocephalus africanus* L. (ERI); *Thymus capitatus* (L.) Hoffmanns. & Link (TCP); *Citrus reticulata* Blanco var. 'Clemenules' (TAN) and *Citrus limon* (L.) Osbeck var. 'Eureka' (LEM). Each EO was supplied to pots containing 560 g of soil at three different doses (low, medium, high). After 15, 30, 90, 120 days the supply of EOs, soils were destructively analyses for microbial biomass carbon (MBC) and microbial respiration. EOs extracted from *E. camaldulensis* (EUC), *C. limon* (LEM) and *T. capitatus* (TCP), at the highest concentration decreased MBC up to 30 days since their addition, with no further effects at two last samplings. EOs extracted from ERI and TAN did not affect MBC. Soil respiration was not affected by any experimental factor, whereas the metabolic quotient was increased by EO extracted from TCP. Our results suggested that essential oils with allelopathic potential extracted from mediterranean plants can negatively affect soil microorganisms and, consequently, their use as herbicides should take into account these findings.

Key words: soil quality, herbicides, essential oils, bioindicators

INTRODUCTION

Weeds are undesired in agriculture and in recreational green areas, as well as in protected spaces (Andonian *et al.*, 2011; Odom *et al.*, 2003). As a consequence, weed management is a common practice in cropped soils, while only occasional in natural ones.

Five different methods are available either to manage or to control weeds: cultural (crop reinforcement against weeds), mechanical and physical (tillage, burning, hand-removing etc.), biological or biochemical, biotechnological and chemical (Rhoads *et al.*, 1989; Duke *et al.*, 2003). Among these five methods, chemical weed management has been preferred by farmers due to the easiness in their use, large number of weed species controlled and, fast and long lasting effect. However, chemical herbicides may affect ecosystem functioning by pollution which may damage both environment and human and animal health (Hatcher and Melander, 2003). Moreover, the overuse of synthetic herbicides can also select herbicide-resistant weeds (Heap, 2014), affect both soil organic matter mineralisation and microbial community composition (Haney *et al.*, 2000; Lancaster *et al.*, 2010).

As alternative to the traditional synthetic herbicides, natural herbicides are being developed, based on allelopathic substances (allelochemicals) deriving from plants or microorganisms, with some of them already available on the market (Bhowmik and Inderjit, 2003). Due to their low environmental persistence and their different mode of action, natural herbicides are more sustainable, thereby preventing selection of herbicide-resistant weeds (Dayan *et al.*, 1999; Dayan *et al.*, 2009; Dubey, 2010; Reigosa *et al.*, 2006) Moreover, the European Union suggested the use of integrated pest management as an alternative to traditional pest management in order to reduce risks and impacts of synthetic herbicide on human health and environment. Integrated pest management includes the use of natural products (such as essential oils) to inhibit weed growth and simultaneously enhance soil biological quality, thereby repelling pests and preventing plant pathologies (Koul *et al.*, 2008; Moss, 2010).

Allelochemicals are secondary metabolites produced by living organisms to interact (either positively or negatively) with other species. Allelopathy plays a role in regulating population abundance of co-existing plants, insects, fungi and microbes in ecologically mature communities.

The use of essential oils (EOs) extracted from plants with negative allelopathic interactions (phytotoxic effects) against weeds is worldwide established (Bhadoria, 2011; Reigosa *et al.*, 2006). *Eucalyptus camaldulensis* Dehnh and *Eriocephalus africanus* L. EOs showed herbicidal effects on common target weeds (Verdeguer *et al.*, 2009). Moreover, EOs interactions with microbial communities are widely applied in medicine, food preservation and pest management (Behdani *et al.*, 2012; Koul *et al.*, 2008; Lv *et al.*, 2011; Palazzolo *et al.*, 2013). EOs extracted from *E. camaldulensis* have been used in pharmacology as antimicrobial agents and in food industry as additives (Kalemba *et al.*, 2003; Solórzano-Santos and Miranda-Novales, 2012). *E. africanus*, an endemic species from South Africa, has been traditionally used to treat dermal and gastrointestinal infections, as antipyretic and analgesic on mammals and as antimicrobial in food preservation (Amabeoku *et al.*, 2000; Salie *et al.*, 1996; Viljoen *et al.*, 2006). EOs extracted from lemon and tangerine tissues have well-known antimicrobial and antifungal effects (Palazzolo *et al.*, 2013). Moreover, *Thymus capitatus* (L.) Hoffmanns & Link is commonly used as spice in Mediterranean diet for its flavour and preservative properties (Lv *et al.*, 2011). Its EO contains mainly thymol and carvacrol, that negatively interact with many living organisms, even pests and weeds (Koul *et al.*, 2008; Regnault-Roger *et al.*, 2012), human pathogens (Dutta *et al.*, 2007) and phytopatogens (Behdani *et al.*, 2012; Tabti *et al.*, 2014).

Despite the great number of studies on EOs microbiocidal and herbicidal properties, few of them have investigated their effects on soil microorganisms. Such an aspect is of great concern since soil microorganisms are the most responsive parameters for soil quality and, at the same time, play a major role in soil fertility and resilience (Laudicina *et al.*, 2012).

The aim of this study was to assess the potential phytotoxic effects of EOs extracted from different mediterranean plants on soil microbial biomass and activity.

MATERIALS AND METHODS

Experimental design

A pot experiment was established on 10 August 2014, using the topsoil (0-10 cm) collected in the inter row zone of a tangerine orchard (Vall D'Uixó, 39° 46' 28" N; 0° 16' 5" O). The main chemical and physical properties of the soil were determined (Table 1).

EOs were extracted by hydrodistillation in a Clevenger-type apparatus from fresh and mature leaves of *Eucalyptus camaldulensis* Dehnh (EUC); *Eriocephalus africanus* L. (ERI); *Thymus capitatus* (L.) Hoffmanns. & Link (TCP); *Citrus reticulata* Blanco var. 'Clemenules' (TAN) and *Citrus limon* (L.) Osbeck var. 'Eureka' (LEM).

A total of 192 plastic pots (10 cm ø; 15 cm height), were filled with 560 g of soil sieved at ø <1.2 cm. The soil was brought to 2/3 of its water holding capacity (WHC) and left over night. The day after, the treatments with EO at concentrations of 1, 2 and 4 mL L^{-1} were applied in a volume equivalent to 1/3 of the soil WHC (Table 2). To emulsify the essential oils in water, Fitoil, a biological coadjuvant composed by soybean oil, was used, at the dose 1 mL L^{-1}. A control treatment with Fitoil (CTR 1 mL L^{-1}) was included. After the addition of EO emulsions, pots were incubated in a greenhouse for 120 days at 30°C and 70% air humidity. During the incubation water loss by evaporation was reintegrated using tap water so that the soil was maintained at 50% of its WHC by monitoring the weight of pots and eventually adding the required amount of water. Such control was carried out two times a week. On days 15, 60, 90 and 120 since the beginning of incubation, three pots per treatment were destructively sampled for soil analyses.

Table 1: Main chemical and physical properties of the soil used in the experiment

Soil properties	
Clay	1.2%
Silt	4.9%
Sand	93.9%
pH	7.5
Electrical Conductivity	1.5 dS m^{-1}
Total N (TN)	0.18 g Kg^{-1}
Total organic Carbon (TOC)	10.5 g Kg^{-1}
TOC/TN	58.3
Total carbonates	12.3%

Table 2. Treatments with EOs applied

Essential oil	**Concentration of EO emulsion**	**μl EO pot^{-1}**	**μl EO g^{-1} of soil**	**Code**
Tangerine	1 mL L^{-1}	80	0.143	TAN_LOW
	2 mL L^{-1}	160	0.286	TAN_MED
	4 mL L^{-1}	320	0.571	TAN_HIGH
Lemon	1 mL L^{-1}	80	0.143	LEM_LOW
	2 mL L^{-1}	160	0.286	LEM_MED
	4 mL L^{-1}	320	0.571	LEM_HIGH
E. camaldulensis	1 mL L^{-1}	80	0.143	EUC_LOW
	2 mL L^{-1}	160	0.286	EUC_MED
	4 mL L^{-1}	320	0.571	EUC_HIGH
E. africanus	1 mL L^{-1}	80	0.143	ERI_LOW
	2 mL L^{-1}	160	0.286	ERI_MED
	4 mL L^{-1}	320	0.571	ERI_HIGH
T. capitatus	1 mL L^{-1}	80	0.143	TCP_LOW
	2 mL L^{-1}	160	0.286	TCP_MED
	4 mL L^{-1}	320	0.571	TCP_HIGH

Chemical and biochemical analysis

Soil samples were air-dried, sieved at 2 mm and stored in sealed polyethylene bags at 4°C prior to biochemical analyses that were carried out within ten days.

Microbial biomass C (MBC) was determined by the fumigation-extraction method (Vance *et al.*, 1987) as described in Laudicina et al. (2011) and the concentration of K_2SO_4-extractable C from non-fumigated soil was assumed as a proxy of available C (Laudicina *et al.*, 2013). Soil respiration (SR) was determined by measuring the cumulative CO_2 evolved during 3 days of soil incubation at 50% of WHC and 22°C as described Laudicina et al. (2015). Metabolic quotient (qCO_2), i.e. the amount of CO_2 emitted per unit of MBC per hour, was expressed as mg CO_2–C g^{-1} MBC h^{-1}.

Statistics

Reported results are means ± standard deviations from three replicates. Data were subjected to two-way analysis of variance (ANOVA) repeated measures (experimental factors: type and concentration of EO). Statistical analysis was performed using Statgraphics Centurion version 15.0 (Statpoint Inc., USA, 2005). Post-hoc Tukey test was used for means comparison at $P<0.05$.

RESULTS

Microbial biomass C (MBC) was affected by EO type and concentration, as well as by their interaction (Table 3). EOs extracted from *E. camaldulensis* (EUC), *C. limon* (LEM) and *T. capitatus* (TCP), at the highest concentration (0.571 µl EO g^{-1} of soil), behaved similarly as decreased MBC up to 30 days since their addition, with no further effects at two last samplings (90 and 120 days). On the other hand, EO from LEM acted peculiarly compared to other ones, since it had no effect on MBC at day 15, while decreasing it at day 30, like EUC and TCP, and being the only EO increasing MBC (at 90 days). None significant differences occurred among treatments 120 days after EOs addition (Figure 1). EOs extracted from ERI and TAN did not affect MBC at any soil sampling time.

Table 3: Two-way ANOVA repeated measures (experimental factors: type and concentration of essential oils) carried out on soil biochemical properties determined at day 15, 30, 90 and 120 since the supply of essential oils (EOs) extracted from five different plants. NS, not significant.

Soil properties	**Type of EO**	**Concentration**	**EO x Concentration**
Microbial biomass C	F=7.5; P<0.001	F=4.8; P<0.016	F=3.9; P<0.002
Extractable organic C	F=37.1; P<0.001	NS	F=14.8; P<0.01
Soil respiration	NS	NS	NS
Microbial quotient	F=12.6; P<0.001	NS	F=7.9; P<0.001

Extractable organic C (C_{extr}) was significantly affected by EOs type and by type x concentration (Table 3). Generally, during the 120 days of incubation C_{extr} decreased in all treatments and no univocal differences comparing treatments to the control were evidenced (Figures 2a, 2b, 2c).

Soil respiration was not affected by any experimental factor, whereas the metabolic quotient (qCO_2), i.e. the amount of CO_2 emitted per unit of MBC per hour, was affected by EO type (F=12.6; P<0.05) and by the interaction EO type x concentration (F=7.9; P<0.05). Only EO extracted from TCP increased the metabolic quotient (Figure 3) and such increases lasted up to 90 days, being generally greatest at the two highest doses of EO supplied.

DISCUSSION

Essential oils extracted from *E. camaldulensis* and *E. africanus* are recognized for their capacity to either limit or avoid weed germination (Verdeguer *et al.*, 2009); citrus EOs are commonly used as microbiocide in food and drugs industry; while *T.capitatus* is a widely studied plant for its use in food industry, medicine and as pesticide.

However, very few studies are available on the effects of these EOs on soil microbial biomass and activity. The reduction of MBC in EUC, LEM and TCP treatments accord to other studies investigating the antimicrobial effects of EOs on microorganisms or in food preservation. Effectiveness of *Eucalyptus* EOs against pathogenic soil-living fungi as *Colletotrichum* *graminicola*, *Phoma sorghina* and *Fusarium moniliforme* has been already reported (Somda *et al.*, 2007). Citrus EOs are active against many groups of microbes and have been used for pest management and food preservation (Palazzolo *et al.*, 2013). Studies carried out on EOs extracted from different citrus cultivars indicate that they are very effective against the Gram-positive bacteria, being lemon cultivars more efficient than tangerine ones (Settanni *et al.*, 2012). In fact, we found also a reduction in MBC in LEM treatment but not in TAN. The absence of a significant MBC reduction in TAN treatment, also at the highest concentration, is reasonable as the used soil was covered by tangerine trees and likely soil microorganisms since long time were exposed to allelochemicals coming from tangerine, thus acquiring an adaptation. The negative effects of EOs extracted from TCP on soil MBC also agree with several studies reporting the antimicrobial effects of EOs from the genus *Thymus* on phytopathogens (Behdani *et al.*, 2012; Tabti *et al.*, 2014), on human pathogens (Dutta *et al.*, 2007) and on foodborne microbes (Cosentino *et al.*, 1999).

The absence of effects of EOs on soil whole respiration disagreed with results reported by Vokou et al. (2006), who found that soil respiration is stimulated by essential oils of aromatic plants rich in carvacrol and/or thymol. From the other hand, our results evidenced an increase of the specific respiration (qCO_2) only in TCP treatment, thus the null response of respiration likely was only apparent due to the concomitant shift of MBC. The qCO_2 represents the quantity of substrate mineralised per unit of MBC and per unit of time. In general, in unsteady ecosystems the qCO_2 value increases in relation to more stable ecosystems (Dalal, 1998; Laudicina *et al.*, 2012). Such an increase may be due to several reasons: new input of fresh substrates C, response of the microorganisms to adverse conditions, predominance of the zymogene flora (r-strategists) over the autochthonous one (K-strategists), or alteration of the bacteria/fungi ratio since they have different carbon use strategies (Dilly and Munch, 1998). Here we can exclude the first hypothesis, i.e. input of fresh substrates C as extractable C did not any increase as qCO_2 did, whereas changes in microbial community structure may be hypothesised.

CONCLUSIONS

Results showed that upon five essential oils used only that extracted from *T. capitatus* had negative impact on both soil microbial biomass and activity. Indeed, Thymus EO reduced microbial biomass C and also the efficiency in using organic C substrates. EOs extracted from lemon and *E. camaldulensis* decreased only soil microbial biomass without affecting its activity, whereas those extracted from *E. africanus* and tangerine did not affect either microbial biomass or activity.

Our results suggested that EOs extracted from EUC, LEM and TCP negatively affected soil microbial biomass C of tangerine soil and their use as herbicides should take into account these findings. Moreover, essential oil extracted from *T. capitatus* negatively affected the carbon use efficiency of the soil microorganisms.

Further studies are needed to understand if the decrease in C use efficiency could be ascribed to changes in microbial biomass and community structure.

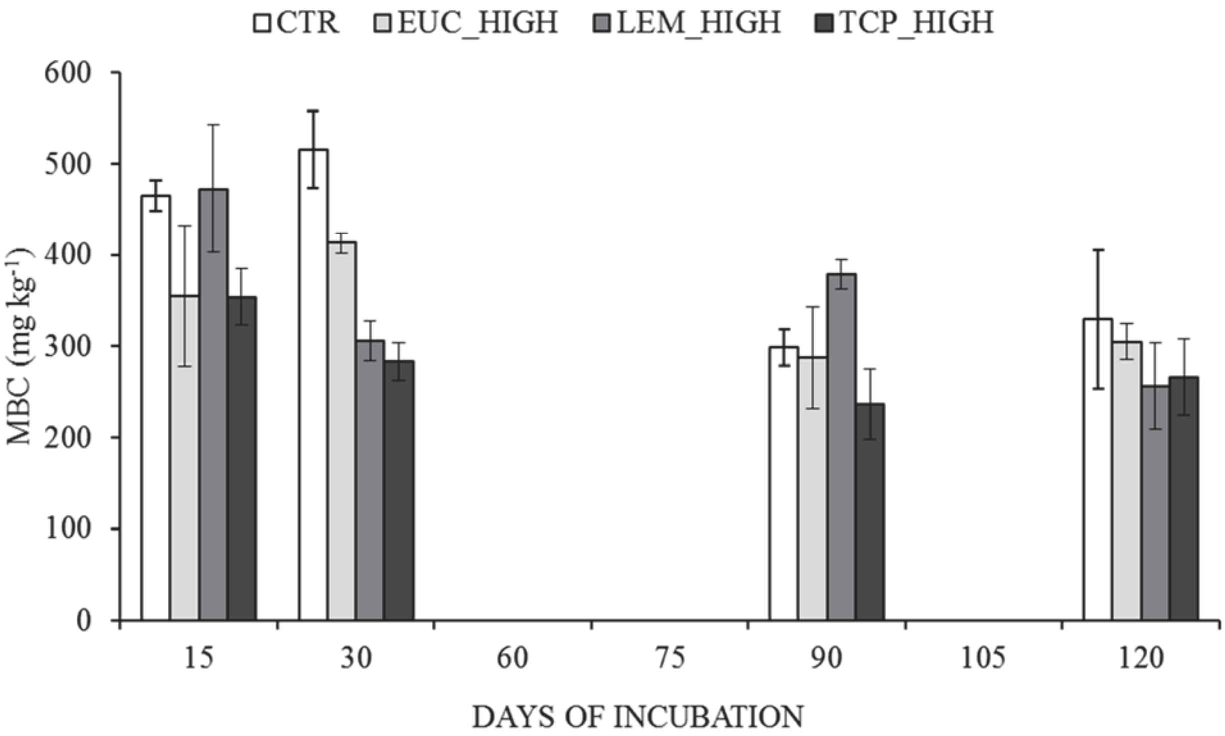

Figure 1. Microbial biomass C (MBC) dynamics after the supply of essential oils at the highest concentration (0.571 µl EO g^{-1} of air dried soil). Only treatments showing significant differences compared to the control (CTR) are reported. Bars are standard deviations (n=3).

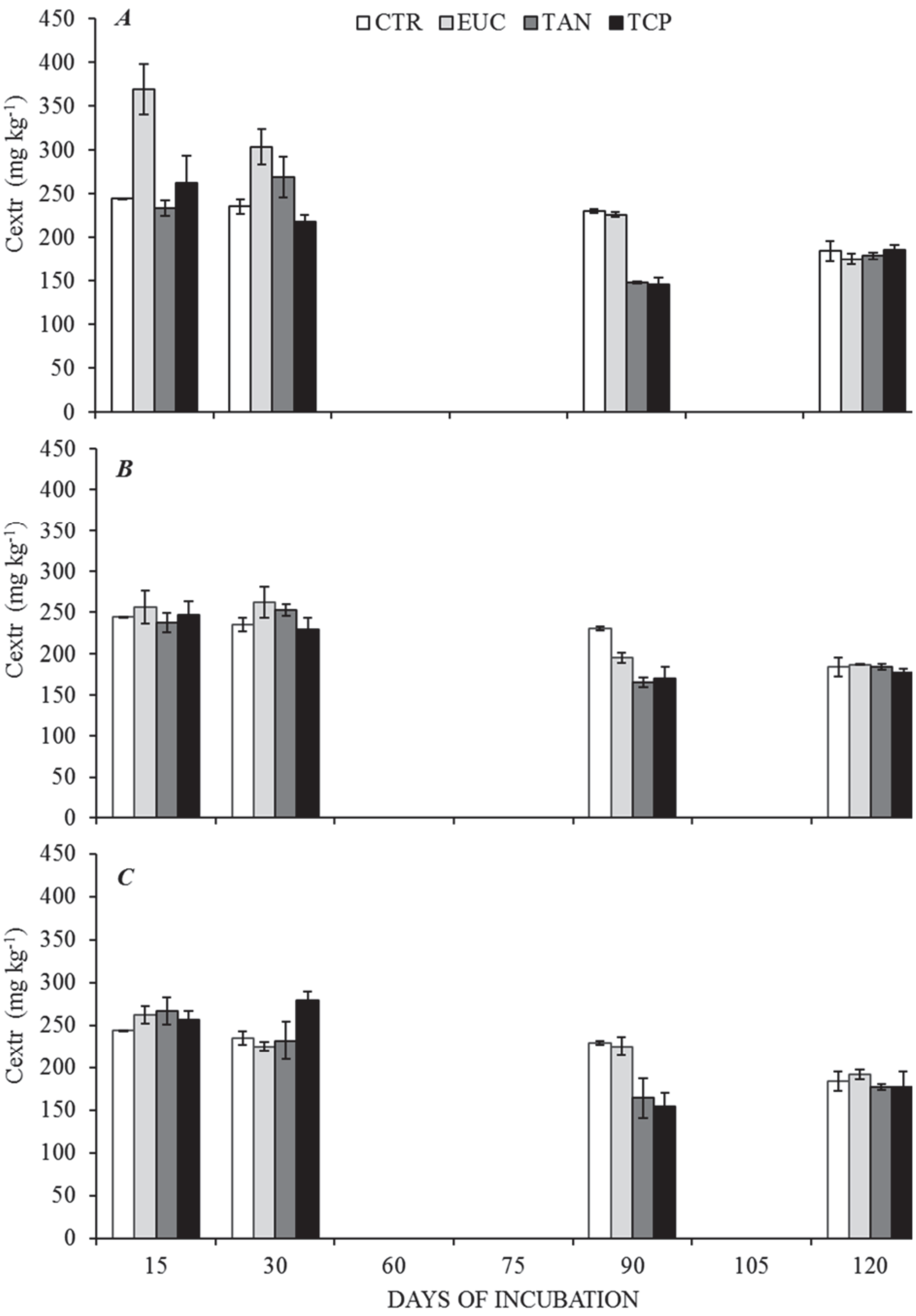
A
B
C
□CTR ▨EUC ▨TAN ■TCP
Cextr (mg kg-1)
0
50
100
150
200
250
300
350
400
450
15
30
60
75
90
105
120
DAYS OF INCUBATION

Figure 2: Extractable organic carbon (Cextr) dynamics after the supply of essential oils at low (a), medium (b) and high (c) concentrations. Only treatments showing significant differences compared to the control (CTR) are reported. Bars are standard deviations (n=3).

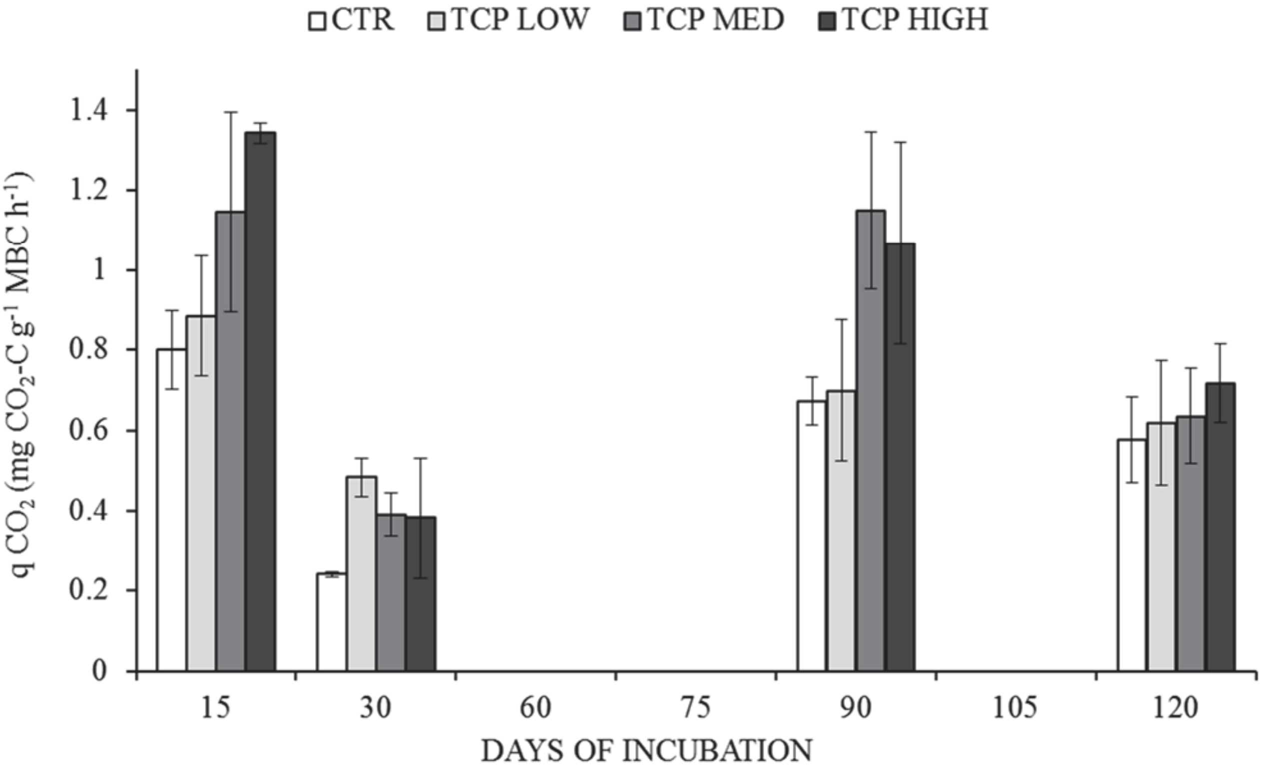

Figure3. Metabolic quotient (qCO$_2$) dynamics in TCP treatment Only treatments showing significant differences compared to the control (CTR) are reported. Bars are standard deviations (n=3).

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