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Eucalyptus globulus Leaves Incorporated as Green Manure for Weed Control in Maize

Carolina G. Puig, Lorena Álvarez-Iglesias, Manuel J. Reigosa, and Nuria Pedrol*

The use of eucalyptus leaves for weed control in maize-based cropping systems is proposed. Aqueous extracts of eucalyptus are known to exert phytotoxicity on many weeds and crops, but there is also experimental evidence of the relative tolerance of maize. Based on in vitro dose-response bioassays of leaf aqueous extracts, we conducted greenhouse pot experiments testing incorporated eucalyptus leaves as green manure. The phytotoxic effects were tested on the germination, establishment and growth of maize and some representative accompanying weeds, in comparison to the PRE herbicide metolachlor. Eucalyptus fresh leaves incorporated into the soil as green manure at 1 and 2% w/w reduced the emergence of the dicot weed species redroot pigweed and black nightshade. After one month of incorporation, both doses reduced aerial biomass >94% two monocot weed species (barnyardgrass + large crabgrass) with respect to the eucalyptus-free pots, and around 80% for the small seeded dicots. Although the aerial biomass of maize was reduced by 33%, the final relative yield of maize biomass with respect to the untreated control increased by 37%. On the assessment of the temporal phytotoxic effects, the reduction of aerial biomass in maize could be overcome by adopting a relay-planting of maize after 12 to 15 days from eucalyptus incorporation. Our results constitute evidence that the incorporation of *E. globulus* residues to soil could be a feasible practice to reduce the reliance on synthetic herbicides in maize-based cropping systems.

Nomenclature: S-metolachlor; barnyardgrass, *Echinochloa crus-galli* (L.) P. Beauv. ECHCG; black nightshade, *Solanum nigrum* L. SOLNI; eucalyptus, *Eucalyptus globulus* Labill. EUGL; large crabgrass, *Digitaria sanguinalis* (L.) Scop. DIGSA; redroot pigweed, *Amaranthus retroflexus* L. AMARE; maize, *Zea mays* L.

Key words: Bioherbicides, *Zea mays*, phytotoxicity, dose-response, pot experiment, relay-planting.

In agriculture, the widespread use of synthetic herbicides for weed control has resulted in many problems such as persistence in soil, development of herbicide-resistant weed populations, and environmental pollution. There is a worldwide effort to reduce the amount of synthetic chemicals used in plant production by introducing modern biological and ecological methods for pest control, e.g., alternatives based on natural products (Duke et al. 2002). One of the possible strategies is the utilization of the chemical interactions between plants for weed control.

On the basis of the allelopathic nature of different species of the genus *Eucalyptus* (Myrtaceae) (e.g. Souto et al. 1994; Zhang and Fu 2009), the pesticide potential of their essential oils and constituents (a complex mixture of monoterpenes and sesquiterpenes, aromatic phenols, oxides, ethers, alcohols, esters, aldehydes and ketones) has been studied (e.g., Batish et al. 2008; FAO 1995; Zhang et al. 2010). There are also reports of phytotoxicity of eucalyptus aqueous extracts, a preliminary requirement to consider a plant material as a potential bioherbicide (Xuan et al. 2005). Evidence of bioactivity on weeds, crops, and wild plant species were reported from many authors (Babu and Kandasamy 1997; Bagavathy and Xavier 2007; Djanaguiraman et al. 2002; Padhy et al. 2000; Pawar and Chavan, 2007; Sasikumar et al. 2002; Willis 1991; Yamagushi et al. 2011; Zhang and Fu 2010). All these reports on the bioherbicide potential of eucalyptus are mostly based on in vitro bioassays. In nature, however, soil plays an essential role in the fate of the released bioactive compounds, so that many of the compounds claimed to be allelochemicals would have little or no biological activity on plants rooted in soil, because of their instability, rapid degradation by microbes, or other interactions with soil (Duke 2010). Valuable field approaches deal with the potential use of allelopathic plants as cover crops or applied to the soil as green manure or mulch (Bhowmik and Inderjit 2003; De Albuquerque et al. 2011; Fujii 2001; Khanh et al. 2005; Xuan et al. 2005). Winter cereals and legumes have received the most attention for their effects on weed suppression and crop development (Campiglia et al. 2012; Cherr et al. 2006; Dhima et al. 2006; Kruidhof et al. 2009). Only a few greenhouse and field studies point out the herbicide potential of eucalyptus. El-Rokiek and Eid (2009) showed that the aqueous extracts of lemon-scented gum (*Eucalyptus citriodora* Hook.) applied to soil reduced the dry weights of wild oat. Zhang and Fu (2009) examined the allelopathic effects of the addition of Timor mountain gum (*E. urophylla* S.T. Blake) leaf litter on the establishment of mixed stands with native species. El-Rokiek et al. (2011) effectively confirmed the phytotoxicity of eucalyptus leaf powder used as surface mulch in a pot experiment, thus obtaining reductions of bermudagrass [*Cynodon dactylon* (L.) Pers] and junglerice [*Echinochloa colona* (L.) Link] dry biomass at concentrations from 40 to 100 g kg^{-1} of soil. These amounts are, however, much higher than the recommended for practical application of crop residues (Xuan et al. 2005).

Despite the phytotoxic effects found on many weeds and crops, evidence of a relative tolerance of maize to *Eucalyptus* sp. aqueous extracts has been reported (El-Khawas and Shehata 2005; Lisanework and Michelsen 1993; Moradshahi et al. 2003; Mubarak et al. 2009). Interestingly, Viera and Schumacher (2011) confirmed the adequacy of intercropping the hybrid *E. urophylla* × *grandis* and maize in an agroforestry system. Espinosa-García et al. (2008) found minimal inhibitory effect of soil from grand eucalyptus (*E. grandis* Hill ex Maiden), *E. urophylla* and *E. grandis* × *urophylla* plantations on the germination and early growth of maize if compared to other crops.

Maize is a widespread row crop with a high demand for weed control in its initial growth phases. This is typically done in Europe using two herbicide applications in PRE and POST

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* PhD Student, PhD Student, Professor, and Assistant Professor, respectively, Department of Plant Biology and Soil Science, laboratory of Plant Ecophysiology, University of Vigo. Campus As Lagoas-Marcosende, 36310-Vigo, Spain. Corresponding author's E-mail: pedrol@uvigo.es

(Meissle et al. 2010; Vasileiadis et al. 2011). If PRE weed control is optimized, the need for POST measures may be reduced. Reduction of pesticide use and risk is one of the key issues of the European Union's agenda for agriculture with Integrated Pest Management becoming compulsory in the EU by the end of 2014 (European Parliament 2009). Green maize for silage has an impact on a key sector of dairy livestock production in the Atlantic and Cantabrian coast of Spain, where it is either grown as continuous maize or in rotation with other crops in nonirrigated systems. In this region, eucalyptus plantations often border on maize fields. In Galicia (north-western Spain), from a total area of 29,434 km^2 Tasmanian blue gum (*E. globulus* Labill.) pure plantations cover around 175,000 ha (MARM 2006). Wood is used mainly to produce cellulose pulp and, secondly, panels and boards. However, despite the evidence of the allelopathic nature of *E. globulus* and the relative tolerance of maize, studies on the use of the abundant eucalyptus harvest residues as green manure for weed control in agroecosystems are absent in literature. This may be a new sustainable prospect "for using the eucalyptus in the twenty-first century as an industrial plantation tree and as a component of farming systems in the rural landscape" (Turnbull 1999), as well as to reduce the reliance on pesticides in maize-based cropping systems (Vasileiadis et al. 2011).

The objectives of this research were (1) to test the in vitro phytotoxic effects of aqueous extracts of fresh *E. globulus* leaves on maize and two representative weeds, in order to set the concentration in soil that would allow a significant weed control, and (2) to determine, under greenhouse conditions, the effects of *E. globulus* fresh leaves incorporated into the soil as green manure on the establishment and early growth of maize and some of the most problematic weeds in maize-based cropping systems, in order to probe the feasibility of the use of eucalyptus harvest residues as a potential tool for field weed control.

Materials and Methods

Plant Material. Fresh adult leaves (also known as *phyllodes*) of *E. globulus* were collected from several 10-yr old plantations surrounding Vigo University campus (Galicia, NW Spain, 42.15°N, 8.43°W) in April 2011. Mean C and N contents on a dry mass basis as determined in triplicate with a Fisons Instruments EA1108 were 49.5 and 1.4%, respectively. Mean PO_4^-, K^+, Mg^{2+}, Mn^{2+} and Ca^{2+} values determined by ICP-OES (Perkin Elmer Optima 4300DV) were 1.1, 7.0, 1.6, 1.5, and 9.4 mg g^{-1}. The dry weight/fresh mass ratio was obtained by drying fresh material aliquots of known leaf area at 70 C for 72 h, specific leaf area (SLA) mean values ranging between 37 and 40 cm^2 g^{-1}. For the greenhouse experiments, fresh plant material was frozen at −80 C immediately after harvest, and maintained at 4 C during the assembly of the experiments.

Laboratory Experiments. *Preparation of aqueous plant extracts*. Fresh eucalyptus adult leaves were slashed in 1cm^2-sized pieces immediately after collection. Extractions were performed in 2-L Erlenmeyer flasks by immersing 350 g of plant material in 1500 ml distilled water. Flasks were left in the dark at room temperature for 24 h., being gently soaked every 6 h. In that way, we obtained a plant dry weight/distilled water volume ratio of 66.7 g L^{-1}. The aqueous extract was vacuum filtered through a cellulose membrane (0.45 µm pore size) to clear impurities and then filtered through 0.2 µm to sterilize the extract. The extract was then frozen at −20 C in sterile plastic bottles until bioassayed.

In vitro dose-response bioassays. Crude extract was successively diluted in distilled water at 100, 50, 25, 10, and 0% (v/v) to get five final concentrations, those corresponding to 66.7, 33.3, 16.7, 6.7, and 0 g L^{-1} on a dry mass basis. Values for pH (Crison MicropH 2001), electrical conductivity (EC, Crison CDTM-523), and osmolarity (Gonotec OSMOMAT 030 cryoscopic osmometer) were measured for each solution. Dose-response assays for seed germination, radicle and shoot growth, were carried out for maize cv. 'Anjou 387' (Limagrain Ibérica S. A., Elorz, Spain) as target crop and redroot pigweed and barnyardgrass from Herbiseed© (Twyford, England, United Kingdom RG10 0NJ) as representative dicot and monocot weed species of significance in European maize production (Meissle et al. 2010). Seeds of weed species were previously synchronized by soaking in distilled water at 4 C for 15 d. For germination tests, maize seeds were incubated in 13 cm diameter Petri dishes at a rate of 15 seeds per dish placed on a filter paper layer wetted with 10 ml of solution. Weed seeds were incubated in 6-well dishes, at a rate of 15 seeds per well placed on a filter paper layer wetted with 600 µL of solution. Seeds were incubated in the dark at 27 C for maize and 35 C for redroot pigweed, and at 26/16 °C, 16/8 h light/dark in the case on barnyardgrass. The number of germinated seeds (rupture of seed coats and emergence of radicle $\geq$ 1 mm; Mayer and Poljakoff-Mayber 1963) was counted every 24 h until no further seeds germinated. The total percentage of germinated seeds (Gt) was calculated from the cumulative germination data. For growth bioassays, ten pre-germinated seeds (radicle length between 1 and 3 mm; Mayer and Poljakoff-Mayber 1963) were used as described above. After 48 h of incubation, radicle and shoot lengths were measured and values were expressed as a percentage of the control. The synthetic PRE herbicide S-metolachlor 960 g L^{-1} (Dual Gold®, Syngenta Agro S.A., Madrid, Spain) was used as positive control at 0.153 g L^{-1} (540 µM) in such a way attaining the label dose (LD, 0.96 kg ha^{-1}). Each treatment was replicated three times.

Greenhouse experiments. *Greenhouse experiment 1: Assaying weed control by eucalyptus green manure*. A greenhouse pot experiment was carried out in June 2011, in order to measure the effects of eucalyptus adult leaves incorporated into the soil on the establishment and early growth of maize and some representative accompanying weeds. A sandy-loam top-soil (A horizon) was collected from an agricultural field devoted to ryegrass-forage maize production for 30 yr and then left fallow over the last three yr. Soil physicochemical characteristics were pH (1:2.5 H_2O) 4.6; EC < 0.13; organic matter 3.12%; total N 0.17%; concentrations of Ca^{2+}, K^+, Mg^{2+}, Na^+ and P^{3-} 234, 71, 23, < 15 and 115 mg kg^{-1}, respectively; and a maximum water retention capacity (WRC) of 316 ml kg^{-1} DW. Fresh eucalyptus leaves slashed in 1 cm^2 size pieces were assayed at two different amounts in soil: 2 and 1% (w/w) on a dry mass basis, suited to attain concentrations of 66.7 and 33.3 g dry weight per L of the maximum WRC of the soil. Five-liter plastic pots (20-cm-diam) were filled with 4000 g soil thoroughly mixed with 240 or 120 g of the fresh plant material. Control treatments consisted of eucalyptus-free pots (negative control) as well as pots watered once at the beginning of the assay with metolachlor at LD (positive control), which half life in soil under aerobic, warm conditions is 30 to 50 d (Böger 2003). Control pots were supplemented with drinking straws cut in 1-cm pieces, thus mimicking the padding effect of the same volume of plant material incorporated into the soil (Wuest et al. 2000). Patent PK (K+S KALI GmbH Kassel, Germany) (P_2O_5 12%, K_2O 15%, MgO 5%) was added to every pot at a dose of 800 kg ha^{-1}. Nitrogen basal dressing or calcium amendment was discarded in order to observe any fertilizing effect of the eucalyptus green manure. Each treatment was replicated three times. Pots were watered to maximum WRC and then weighted.

Each pot was sown at 2 cm depth with five equidistant seeds of maize and five seeds of field bindweed (*Convolvulus arvensis* L.), and then evenly seeded with 24 mg of seeds of any of the following species: redroot pigweed, barnyardgrass, common purslane (*Portulaca oleracea* L.), black nightshade, and large crabgrass, thus achieving the seed bank densities of small seeded weeds in infested fields of corn (i.e. 6 g m^{-2} ; Dhima et al. 2009). All weed seeds were purchased from Herbiseed©. Weeds and maize seedling emergence was recorded every other day until control pots were crowded (10 d after sowing). Pots were weighed every other day and the water lost by evapotranspiration (ET) was replaced. Thirty days after sowing, the final number of emerged alive seedlings was counted. Plants were harvested by cutting at soil level and then dried at 70 C for 72 h to obtain the aerial biomass (g DW) production for each species. Maize yields were calculated as yield (%) = [maize aerial biomass / (maize aerial biomass + total weed aerial biomass)] × 100. After harvest, soil pH and EC from each pot were determined in a soil : water ratio of 1 : 2.5. The experiment was carried out under homogeneous greenhouse conditions for all plants (natural light, T ≤ 26 C maintained by a cooling system).

Greenhouse Experiment 2: Assessing Temporal Phytotoxic Effects of Eucalyptus Green Manure. On the basis of the results obtained from the greenhouse experiment 1, another pot experiment was carried out in July 2011 in order to measure the evolution of the phytotoxic effects of eucalyptus leaves incorporated into the soil, on the germination, establishment, and early growth of maize and two representative weeds: redroot pigweed and barnyardgrass. Plastic pots of 1 L (13 cm diam) were filled with 800 g soil thoroughly mixed with 50 g eucalyptus leaves as described above, at a dose of 2% (w/w). Control treatments consisted in eucalyptus-free pots as well as pots watered once at the beginning of the assay with metolachlor at LD. Pots for 10 sowing times per treatment were concurrently prepared and watered. Pots for the first sowing time were immediately sown with 25, 25, and 15 seeds per pot of redroot pigweed, barnyardgrass and maize, respectively. A new sowing event was carried out every third day. All treatments and times of sowing were replicated four times. Pots were weighed every other day and the water lost was replaced. Ten days after each sowing time, maize and weed seedlings were carefully dug up and counted, and maize roots cleaned of soil particles. Then, the number of germinated seeds, the root and shoot lengths for each maize seedling, the shoot length for weeds, and the total root and aerial biomasses per species and pot were recorded. Post-harvest soil pH and EC measurements and environmental conditions during the experiment were as described above.

Statistical analysis. Replicated experiments were conducted in a completely randomized design. Data were firstly tested for normality by Kolmogorov-Smirnov test and for homogeneity of variances by Levene's test. When variances were homogeneous, data were analyzed by one-way ANOVA, and LSD test ($P = 0.05$) for post hoc multiple comparisons. In the case of heterocedasticity, data were analyzed by Kruskall-Wallis H test and Tamhane's T2 ($P = 0.05$) for post hoc multiple comparisons. For the greenhouse experiment 2, data obtained for each parameter and sowing time were expressed in percentage with respect to the negative and the positive control, and then compared to them by independent samples Student's t-test ($P = 0.05$). The data collected from dose-response study was subjected to log transformations and polynomial models were fitted to data. ED_{50} and ED_{80} values (concentrations required to obtain 50 and 80% inhibition, respectively) were calculated from the generated dose–response curves. Statistical analyses were performed using the IBM SPSS Statistics 19 software.

Results and Discussion

In vitro dose–response bioassays. Values of pH for the increasing concentrations of eucalyptus aqueous extracts ranged between 4.74 and 4.40. Their EC values were 0.16, 0.40, 0.70 and 1.37 dS m^{-1} for 6.7, 16.7, 33.3 and 66.7 g DW L^{-1}, respectively. Dhima et al. (2009) stated that EC values lower than 2 dS m^{-1} do not explain germination or growth inhibition of target species per se. An et al. (1997) suggested that, although the EC has some correlation with phytotoxicity, the osmotic effect of extracts is not sufficient to account for the observed phytotoxicity. In fact, our osmolarity measurements on the different dilutions of the eucalyptus crude extract gave very low values ranging from 0.006 to 0.064 osmol kg^{-1}, all of them lower than that of S-metolachlor at 0.153 g L^{-1} (0.099 osmol kg^{-1}). So, any inhibitory effect observed in our dose–response assays could a priori be attributed to phytotoxic effects.

From the ANOVA of the effects of eucalyptus aqueous extracts (data not shown), maize germination was not inhibited ($P > 0.05$). Mean Gt values ranged between 100 and 69%, with no differences among the different doses and metolachlor ($P > 0.05$). Only at the highest assayed concentration (66.7 g DW L^{-1}), radicle length was lower ($P < 0.05$) than the control, exerting similar inhibition than S-metolachlor (47% inhibition). ED_{50} and ED_{80} calculated values were out of the range of bioassayed doses (Table 1). A significant dose–response effect (ANOVA, $P < 0.001$) was observed towards redroot pigweed germination and growth, the highest bioassayed concentration being similar to S-metolachlor in radicle length inhibition (86 and 88%, respectively). Eucalyptus aqueous extract was innocuous for barnyardgrass germination, but inhibited radicle growth on a dose–response relationship similar to that described for redroot pigweed. Moradshahi et al. (2003) also reported extreme radicle growth reduction of barnyardgrass exposed to *E. camaldulensis* aqueous leaf extracts at 100 g fw L^{-1}. In

Table 1. *Eucalyptus globulus* leaf aqueous extracts ED values for germination besides radicle and shoot length of maize, redroot pigweed and barnyardgrass.

Target species	Response	ED_{50} [a]	ED_{80} [b]
		g dw L^{-1}	g dw L^{-1}
maize	Total germination	o.r.[c]	o.r.[c]
	Radicle length	o.r.[c]	o.r.[c]
	Shoot length	o.r.[c]	o.r.[c]
redroot pigweed	Total germination	20	40.9
	Radicle length	5.1	45.1
	Shoot length	o.r.[c]	o.r.[c]
barnyardgrass	Total germination	o.r.[c]	o.r.[c]
	Radicle length	4.4	33.9
	Shoot length	o.r.[c]	o.r.[c]

[a] ED_{50}.
[b] ED_{80} = dose required to obtain 50 and 80% germination, radicle or shoot length inhibition, respectively.
[c] o.r. = out of range.

every case, radicle length proved much more affected than shoot length, as reflected from ED values (Table 1).

The introduction of a PRE herbicide as positive control for in vitro bioassays with aqueous extracts or essential oils is rarely cited in the literature (e.g. El-Rokiek and Eid 2009; Grosso et al. 2010). In our work, the utilization of the widely used S-metolachlor at the LD turned out to be extremely useful to establish comparisons. The results of the in vitro assays of the phytotoxicity of aqueous extracts of *E. globulus* leaves seemed promising for the possible use the plant material incorporated into the soil as well.

Weed control by eucalyptus as green manure. The results obtained from the greenhouse experiment 1 are summarized in tables 2 and 3. Total dicot density decreased with respect to the control in pots treated with eucalyptus green manure in the first wk of the assay, similar to that exerted by metolachlor (Table 2). The phytotoxic effects of both eucalyptus doses were similar at d 8 and 10, with 45 to 65% emergence of dicot weeds with respect to the control. Increasing rates of mortality of the established dicots were observed in pots treated with metolachlor from d 8 on. Contrary to dicot weed species, the emergence of monocot weeds was poorly controlled by eucalyptus green manure as compared to metolachlor. On the other hand, maize benefited from the treatment with leaves relative to the eucalyptus-free pots, attaining full emergence at d 10 after sowing. Batish et al. (2004) described minimal effects of *E. citriodora* essential oil on germination of grain crops as well. In general, large seeded crops may better tolerate the residue-mediated changes in the chemical properties of soil caused by the incorporation of green manures (Gallandt 2006).

Table 3 shows the effects of *Eucalyptus* extracts and S-metolachlor on the establishment of weeds and maize at d 30 after sowing. With the exception of field bindweed, which was not affected by any treatment, S-metolachlor applied at the label dose controlled weeds almost completely, including the small amount that could emerge during the first d after sowing (see Table 2). Both doses of eucalyptus green manure controlled redroot pigweed and black nightshade weed densities and aerial biomass production, which were comparable to S-metolachlor. The eucalyptus treatment produced scarce and small weed plants, but external injury symptoms were not apparent. Although the number of common purslane seedlings was enhanced by the green manure, aerial biomass was also less than, but not significantly different from eucalyptus-free pots. As denoted during the first d of the experiment, densities of monocot weeds were not reduced by the incorporation of leaves into the soil. Otherwise, the phytotoxic effects on seedling growth were notable, with reductions of aerial biomass around 72 and 97% relative to control pots for barnyardgrass and large crabgrass, respectively. Unfortunately, despite the promising results of the in vitro bioassays, maize aerial biomass per pot was also reduced by the green manure at both doses, thus demonstrating some crop phytotoxicity independent of the increase in soil EC observed. Nonetheless, if expressed in percentage of maize biomass in the total biomass per pot, maize yields in control pots were widely exceeded by those obtained in any of the treatments for weed control, thus confirming the advantage of maize against weeds under the eucalyptus treatment. On the other hand, in terms of sustainability, the addition of eucalyptus leaves to the pots at the higher dose reduced the total water loss by evapotranspiration compared to the negative and positive control treatments. The decay in soil pH was lower in pots with incorporated plant material, whereas basal limestone dressing would be required to attain optimal slightly acid to neutral pH.

Emergence and growth of seedlings were affected by eucalyptus incorporated into the soil in a species-dependent way. However, a dose–response effect was not observed; the phytotoxicity exerted by the plant material incorporated into the soil was statistically similar at 1 and 2% (w/w). Kruidhof et al. (2008) also observed that doubling the recommended sowing density of certain allelopathic cover crops did not

Table 2. Effects of different doses of *Eucalyptus globulus* leaves incorporated into the soil, or metolachlor at label dose (LD, 0.96 kg ha^{-1}), on the emergence and survival of maize seedlings and the accompanying weeds in subsequent d after sowing in a pot experiment. Within each column and row, mean values of three replicates ± SD. For each d of measurement, distinct letters mean significant differences between treatments ($P \leq 0.05$; LSD or Tamhane's T2 post hoc multiple comparisons).

Variable	Days after sowing	sig. [a]	0% w/w	1% w/w	2% w/w	metolachlor LD
Total dicot weeds per pot (n)	4	***	40.0 ± 7.0 a	6.7 ± 4.0 b	16.0 ± 6.6 b	15.0 ± 6.0 b
	6	*	67.7 ± 14.6 a	28.7 ± 13.1 b	45.3 ± 12.9 ab	24.7 ± 11.6 b
	8	**	76.0 ± 15.7 a	37.7 ± 17.9 bc	51.0 ± 9.6 ab	20.7 ± 3.5 c
	10	***	77.7 ± 11.2 a	35.3 ± 17.9 b	51.0 ± 12.1 b	9.3 ± 4.7 c
Total monocot weeds per pot (n)	4	n.s.	9.0 ± 4.4 a	3.0 ± 1.7 a	5.3 ± 0.6 a	2.3 ± 1.5 a
	6	**	26.7 ± 7.0 a	17.7 ± 7.5 a	24.7 ± 1.5 a	6.3 ± 2.1 b
	8	**	31.3 ± 5.9 a	20.3 ± 7.6 b	26.3 ± 2.3 ab	7.0 ± 3.6 c
	10	**	31.7 ± 5.7 a	20.7 ± 7.1 ab	28.0 ± 5.3 a	9.7 ± 4.2 b
Total maize seedlings per pot (n)	4	n.s.	0.0 ± 0.0 a	0.3 ± 0.6 a	0.0 ± 0.0 a	0.3 ± 0.6 a
	6	*	3.0 ± 1.0 a	3.0 ± 0.0 a	4.3 ± 0.6 b	4.3 ± 0.6 b
	8	n.s.	3.3 ± 0.6 a	4.7 ± 0.6 a	5.0 ± 0.0 a	4.7 ± 5.6 a
	10	*	3.7 ± 0.6 a	5.0 ± 0.0 b	5.0 ± 0.0 b	5.0 ± 0.0 b

[a] sig., significant at *$P \leq 0.05$;** $P \leq 0.01$;*** $P \leq 0.001$; n.s., not significant $P > 0.05$ (ANOVA or Kruskal-Wallis H test).

Table 3. Effects of different doses of *Eucalyptus globulus* leaves incorporated into the soil, or metolachlor at label dose (LD, 0.96 kg ha^{-1}), on the growth of maize and the accompanying weeds in a pot experiment. Within each column, mean values of three replicates ± SD measured at d 30 after sowing. For each variable and/or species, distinct letters mean significant differences between treatments ($P \leq 0.05$; LSD or Tamhane's T2 post hoc multiple comparisons).

Variable	species	sig. [a]	0% w/w		1% w/w		2% w/w		metolachlor LD	
Plants per pot (n)	field bindweed	n.s.	2.00 ± 0.00	a	3.67 ± 0.58	a	1.67 ± 1.53	a	1.667 ± 0.577	a
	redroot pigweed	*	14.67 ± 3.22	a	3.33 ± 1.53	b	8.00 ± 2.65	b	0.00 ± 0.00	c
	black nightshade	*	14.33 ±3.22	a	4.33 ± 2.31	b	0.33 ± 0.58	b	0.00 ± 0.00	b
	common purslane	*	10.00 ± 6.25	a	25.00 ± 11.27	ab	24.00 ± 5.57	b	0.00 ± 0.00	c
	barnyardgrass	***	9.67 ± 1.16	a	8.00 ± 1.00	a	9.33 ± 1.53	a	0.00 ± 0.00	b
	large crabgrass	**	23.67 ± 7.64	a	18.00 ± 7.21	a	20.00 ± 4.58	a	0.00 ± 0.00	b
	Total small seeded dicots	**	39.00 ± 11.53	a	32.67 ± 11.59	a	32.33 ± 8.33	a	0.00 ± 0.00	b
	Total monocots	***	33.33 ± 7.51	a	26.00 ± 7.00	a	29.33 ± 6.03	a	0.00 ± 0.00	b
	maize	n.s.	4.00 ± 1.00	a	5.00 ± 0.00	a	5.00 ± 0.00	a	5.00 ± 0.00	a
Aerial biomass per pot (mg DW)	field bindweed	n.s.	17.73 ± 11.75	a	47.97 ± 19.19	a	25.10 ± 25.99	a	23.13 ± 2.17	a
	redroot pigweed	*	11.20 ± 7.37	a	0.53 ± 0.46	bc	2.17 ± 0.76	b	0.00 ± 0.00	c
	black nightshade	*	34.83 ± 13.12	a	4.27 ± 1.63	b	0.03 ± 0.06	bc	0.00 ± 0.00	c
	common purslane	n.s.	7.27 ± 4.56	a	4.57 ± 2.46	ab	5.23 ± 2.60	a	0.00 ± 0.00	b
	barnyardgrass	*	57.77 ± 28.78	a	15.70 ± 3.30	b	15.60 ± 2.29	b	0.00 ± 0.00	c
	large crabgrass	*	475.77 ± 213.77	a	9.83 ± 4.28	bc	15.83 ± 6.21	b	0.00 ± 0.00	c
	Total small seeded dicots	*	53.30 ± 20.34	a	9.37 ± 2.55	b	7.43 ± 3.41	b	0.00 ± 0.00	c
	Total monocots	*	533.53 ± 236.83	a	25.53 ± 6.57	b	31.43 ± 7.84	b	0.00 ± 0.00	c
	maize	**	1148.23 ± 87.32	a	722.50 ± 101.64	b	802.37 ± 118.90	b	1014.73 ± 69.32	a
total ET (mL) [b]		**	6186.47 ± 76.65	a	6128.83 ± 126.92	a	5882.90 ± 31.58	b	6233.53 ± 97.13	a
yield (%) [c]		*	66.41 ± 10.32	a	89.44 ± 4.38	b	92.85 ± 3.22	b	97.76 ± 0.26	b
soil pH		**	4.38 ± 0.03	a	4.50 ± 0.06	b	4.50 ± 0.04	b	4.37 ± 0.04	a
soil EC (dS m^{-1})		***	0.16 ± 0.03	a	0.22 ± 0.04	a	0.47 ± 0.08	b	0.16 ± 0.01	a

[a] sig., significant at *$P \leq 0.05$;** $P \leq 0.01$;*** $P \leq 0.001$; n.s., not significant $P > 0.05$ (ANOVA or Kruskal-Wallis H test).
[b] total ET (ml), total water loss by evapotranspiration.
[c] yield (%), percentage of maize in the total yield (maize + weeds).

increase the residue-mediated suppression of weed establishment. The dose of 1% (w/w), being equivalent to 33.3 g DW L^{-1} in the pot soil solution (close to the ED_{80} for in vitro redroot pigweed germination and barnyardgrass radicle growth, see Table 1), may have been already enough to achieve the maximum weed control. The adequacy of moderate amounts of harvest residues would moreover make fieldwork easier and cheaper (Miller 2007).

Temporal phytotoxic effects of eucalyptus green manure. The reductions in maize biomass in greenhouse experiment 1 must be taken into account for putative field management of eucalyptus residues for weed control. Consequently, a second greenhouse experiment on the temporal phytotoxic effects of eucalyptus green manure was designed to establish a relay-planting of maize after incorporation of eucalyptus leaves into the soil. Figure 1 describes the effects measured on maize seedlings sown at different days after eucalyptus incorporation (DAI) and compared on a percent basis to the eucalyptus-free control, as well to metolachlor applied at LD. No significant effects were detected on germination at any of the sowing dates. Highly significant stimulatory effects on root elongation with respect to the control were observed on maize sown from 21 DAI on. but compared to metolachlor, the benefits of green manure on seedling root elongation were observed all along the assay, concomitantly with increases in root biomass. As suspected from the results obtained in the greenhouse experiment 1, phytotoxicity was measurable on the shoot length and aerial biomass of maize plants sown from 3 to 9 DAI, effects that were also observed when compared to metolachlor. From 12 DAI on, no inhibition but stimulatory effects were reported. A model of two phases, inhibitory and stimulatory, could be argued for the dynamics of the effects of eucalyptus after incorporation into the soil on maize growth. The former, lasting 9 to 12 d, may be related to the accumulation of volatile compounds, mainly eucalyptol, in the soil atmosphere.

This "volatile phase" would finish as soon as the components of essential oil had volatilized (Batish et al. 2008), or their concentration diminished in the soil pores. In that sense, Moradshahi et al. (2003) reported moderate in vitro effects of aqueous extracts of *E. citriodora* on maize radicle growth if compared to the high inhibition exerted by leaf volatile oils. The following phase, coinciding with maize growth stimulation from d 12 on, would point out the onset of a "soluble phase", i.e., the beginning of decomposition processes and nutrients release and /or possible hormetic effects of low concentrations of phytotoxic metabolites that promote growth enhancement (Belz et al. 2011). This suggests that eucalyptus has some value as a slow release fertilizer to partially replace the starter basal dressing for maize. Madeira et al. (2010) reported mean values of leaves nutrients at harvest time around 13, 1, 5, 2, and 12 mg g^{-1} for N, P, K, Mg, and Ca, respectively, which began to be released into the soil immediately after incorporation of plant material until its complete decomposition in around 1 yr (Guo and Sims 2002). Our values detailed in Materials and Methods are similar, with higher K and lower Ca amounts, and a substantial concentration of Mn (1.52 mg g^{-1} DW). In the monitoring of our assay, EC values about 0.05 dS m^{-1} in the soil amended with eucalyptus were higher than those measured from the negative and positive control pots (data not shown), thus reflecting a continuous release of nutrients and other water-soluble compounds. This led to the maintenance of higher pH values ($P < 0.01$) reaching 5.0 during the "soluble phase", if compared to control pots (pH~4.6). From our results, the loss of maize aerial biomass because of planting the very d of green manure incorporation could be overcome by adopting a relay-planting of 12 to 15 d.

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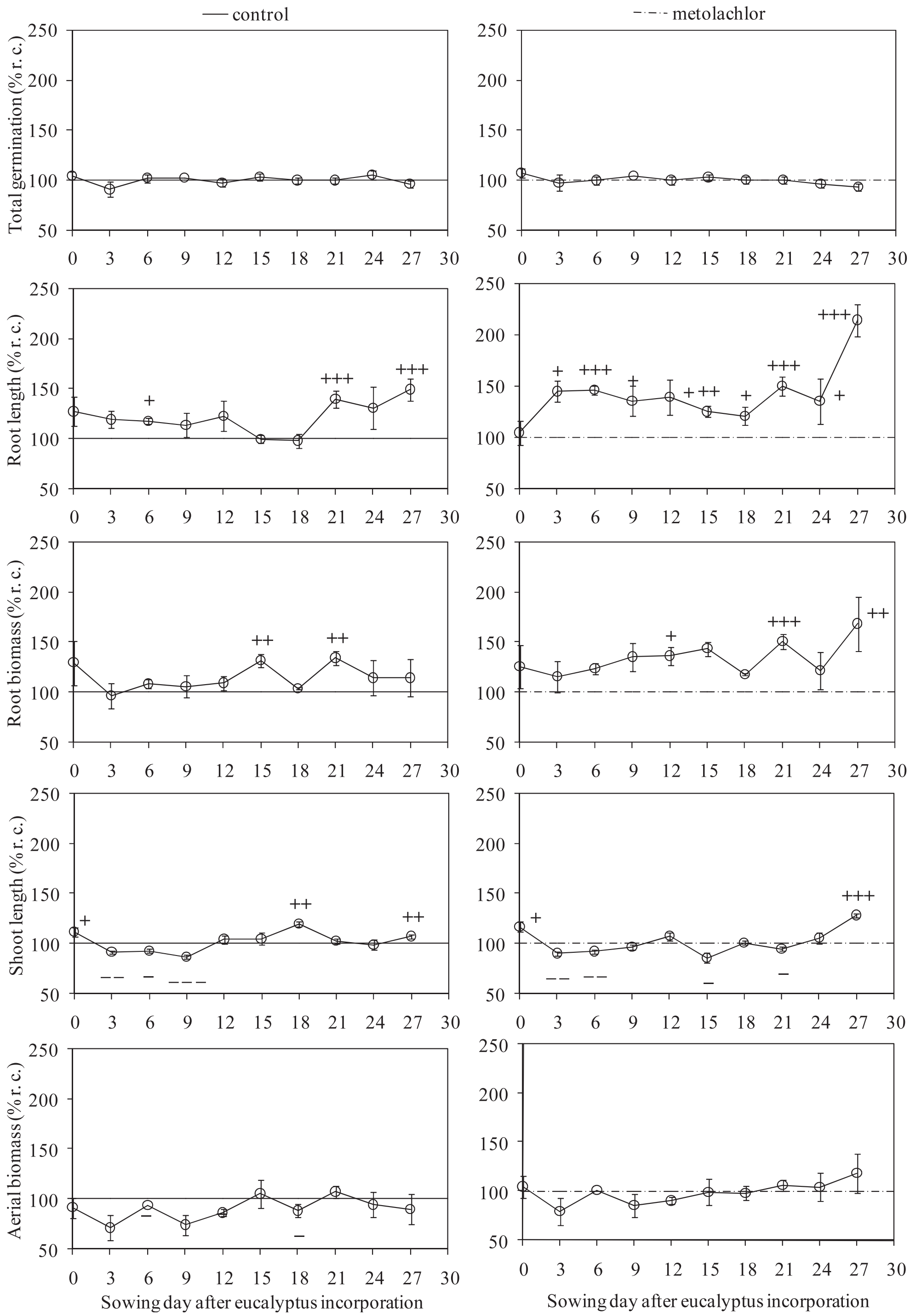

Figure 1. Growth parameters measured in 10 d old maize seedlings sown at different times after incorporation of *Eucalyptus globulus* leaves into the soil. For each parameter and time, signs represent inhibition (−) or stimulation (+) with respect to the control (left) or to metolachlor at label dose, 0.96 kg ha^{-1} (right): one sign, $P \leq 0.05$; two signs, $P \leq 0.01$; three signs, $P \leq 0.001$; no sign, $P > 0.05$ (independent samples *t*-test). Symbols represent mean values of four replicates ± SD. % r.c., percentage with respect to the control; % r.m., percentage with respect to metolachlor.

The temporal effects of eucalyptus leaves incorporated into the soil on redroot pigweed and barnyardgrass are summarized in Figure 2. Inhibition of redroot pigweed germination was above 50% in all the assayed sowing dates. This medium-term continuous effect is significant because seeds in the soil seed bank do not synchronize, but germinate gradually all along the establishment of the crop. Moreover, pigweed control would remain effective before and after the safe period for maize. The scarce growing seedlings were highly stimulated by the eucalyptus green manure compared to the heights of the control seedlings, at least from the onset of the "soluble phase." Finally, redroot pigweed aerial biomass was controlled independent of the sowing DAI, and was practically eliminated during the "volatile phase." General temporal effects expressed with respect to metolachlor were similar in tendency and magnitude (see Supplemental Material for detailed data).

Germination of barnyardgrass was not controlled by eucalyptus green manure. Some stimulatory effects were

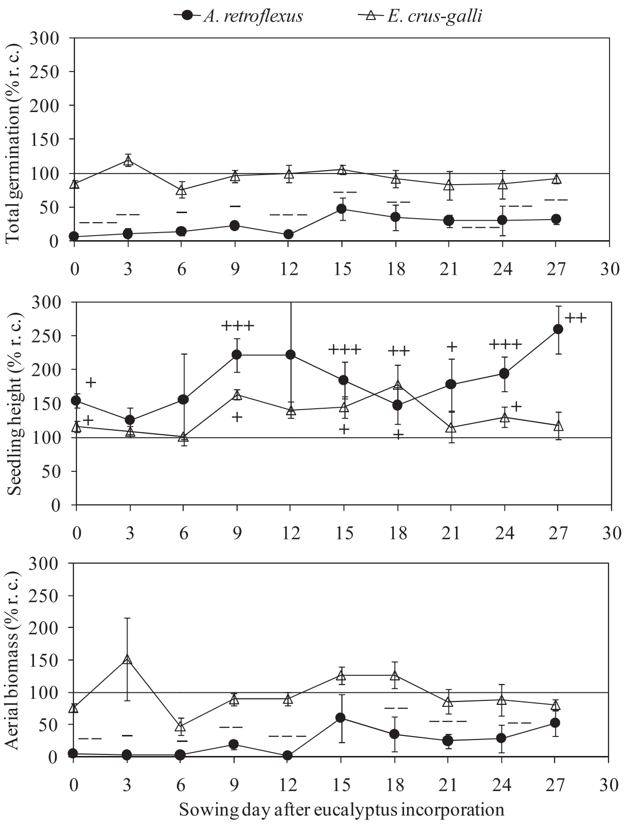

Figure 2. Growth parameters measured in 10 d old redroot pigweed (*Amaranthus retroflexus*) and barnyard grass (*Echinochloa crus-galli*) seedlings sown at different times after incorporation of *Eucalyptus globulus* leaves into the soil. For each parameter and time, signs represent inhibition (−) or stimulation (+) with respect to the control: one sign, $P \leq 0.05$; two signs, $P \leq 0.01$; three signs, $P \leq 0.001$; no sign, $P > 0.05$ (independent samples *t*-test). Symbols represent mean values of four replicates ± SD. % r.c., percentage with respect to the control.

observed on seedling height, with no final effects on biomass production in any of the sowing dates. Batish et al. (2004) described important inhibitory effects of *E. citriodora* essential oil on small seeded dicot weeds such as redroot pigweed, and also minimal effects on barnyardgrass germination. On the experimental use of aromatic plants as green manures, Dhima et al. (2009) discussed weed control on the basis of germination reduction, and stated the absence of any adverse effect on further growth of the survived weeds. In agreement with Kobayashi et al. (2004) and Moore et al. (1994), they attributed the effects to the greater amounts of allelochemicals found in the soil immediately after incorporation of plant residues, which afterwards were reduced drastically by decomposition. In our case, the inhibition of germination would explain the effects on the dicots redroot pigweed and black nightshade found in greenhouse experiment 1, but not those observed in the midterm on barnyardgrass and large crabgrass (see Table 3). The aqueous extract had no effect on barnyardgrass germination (see Table 1), and the emergence and early growth of the monocot weed was unaffected by the eucalyptus green manure (Figure 2), whereas their biomasses were strongly reduced in the medium term (Table 3). Possibly, the release of water-soluble allelochemicals affecting monocot weeds from lignin rich mature leaf tissues (having mean C/N ratios up to 50 ; Guo and Sims 2002, and 36.4 in our case — see Materials and Methods), is attained more gradually and maybe concomitantly to the onset of decomposition and release of nutrients into the soil. In our approach, the maintenance of moisture of soil at warm temperatures would provide high rates of plant residues decomposition and probably favor a continuous release of active phytotoxins. Again, this medium to long term weed control effects of eucalyptus harvest residues must be emphasized in spite of the feasibility of its use at a field scale, although the interactions of environmental factors and management practices would undoubtedly modify the described dynamics (An et al. 2002).

The combination of *in vitro* assays with green manure pot experiments on the phytotoxic effects of *E. globulus* leaves was extremely useful to appraise this abundant resource to be potentially used for weed control at a field scale. *E. globulus* leaves incorporated into the soil as green manure could control the weed species redroot pigweed, black nightshade, barnyardgrass and large crabgrass. Different modes of action are pointed out for target dicots and monocots, through inhibition of germination or effects on medium-term growth, respectively. From our pot experiments, the symptoms of phytotoxicity measured on maize could be overcome by adopting a relay-planting for maize of 12 to 15 d after eucalyptus incorporation, but this period can be different under field conditions depending on the dynamics of residues decomposition. Our results constitute evidence that *E. globulus* leaves incorporated into the soil could be a feasible practice for weed control in sustainable maize-based cropping systems. Of course, transference to field exploitation requires extra greenhouse and field approaches including dose-–response effects on weeds and crops, the assessment of eucalyptus fertilizing value to adjust crop fertilization requirements, the potential inhibition of microbial activity by the release of secondary compounds, the variation of effects depending on eucalyptus seasonal composition or phenological cycle, or in deep studies on the fate of phytotoxins in the soil, amongst others.

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