

Chemical facilitation and induced pathogen resistance mediated by a root-secreted phytotoxin

Balakrishnan Prithiviraj¹, Laura G. Perry^{2,3}, Badhri V. Dayakar² and Jorge M. Vivanco²

¹Department of Plant and Animal Sciences, Nova Scotia Agricultural College, Truro, Nova Scotia, Canada B2N 5E3; ²Center for Rhizosphere Biology and

³Department of Forest, Rangeland, and Watershed Stewardship, Colorado State University, Fort Collins, CO 80523-1173, USA

Summary

Author for correspondence:

Jorge M. Vivanco

Tel: +1 (970) 491 7170

Fax: +1 (970) 491 7745

Email: j.vivanco@colostate.edu

Received: 10 August 2006

Accepted: 30 October 2006

- The flavonol (±)-catechin is an allelochemical produced by the invasive weed *Centaurea maculosa* (spotted knapweed). The full effects of (±)-catechin on plant communities in both the native and the introduced ranges of *C. maculosa* remain uncertain.
- Here, by supplementing plant growth media with (±)-catechin, we showed that low (±)-catechin concentrations may induce growth and defense responses in neighboring plants. Doses of the allelochemical lower than the minimum inhibitory concentration (MIC) induced growth in *Arabidopsis thaliana*; plants treated with 25 µg ml⁻¹ (±)-catechin accumulated more than twice the biomass of untreated control plants. Further, pretreatment of *A. thaliana* roots with low concentrations of (±)-catechin induced resistance to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 in *A. thaliana* leaves.
- Low doses of (±)-catechin resulted in moderate increases in reactive oxygen species (ROS) in the meristems of treated plants, which may have loosened the cell walls and thus increased growth. Experiments with *A. thaliana* mutants indicated that (±)-catechin induces pathogen resistance by up-regulating defense genes via the salicylic acid (SA)/nonexpressor of pathogenesis related protein 1 (NPR1)-dependent pathway.
- Our results suggest that the growth and defense-inducing effects of (±)-catechin are concentration dependent, as (±)-catechin at higher concentrations is phytotoxic, thus suggesting the potential for hormesis to occur in nature.

Key words: allelopathy, *Arabidopsis thaliana*, *Centaurea maculosa*, hormesis, plant–plant interaction.

New Phytologist (2006) doi: 10.1111/j.1469-8137.2006.01964.x

© The Authors (2006). Journal compilation © *New Phytologist* (2006)

Introduction

Plant survival in natural communities depends on the outcome of positive and negative interactions with neighboring plants, microbes, animals and the environment (Hirsch *et al.*, 2003). Two-way interactions such as plant–plant, plant–microbe, and plant–invertebrate interactions have been studied at ecological, biological and biochemical levels (Callaway, 1995; Baker *et al.*, 1997; Stoll & Weiner, 2000; Bais *et al.*, 2003), but more complex interactions comprised of more than two biological components have received less attention. Plants produce chemical signals in

response to pathogen and insect attacks that induce defense responses and alert neighboring plants to the presence of potential pathogens or insect herbivores (Bruin *et al.*, 1992; Shulaev *et al.*, 1997; Kessler & Baldwin, 2001), or that attract insect parasites (Dicke *et al.*, 1990; Bruin *et al.*, 1992; Kessler & Baldwin, 2001; Dicke *et al.*, 2003). Plant–plant and plant–invertebrate communication in response to attacks may often be a case of ‘eavesdropping’ by neighboring plants and invertebrates and may not be intended by the plant under attack; nevertheless, such communication may, by affording an advantage to some organisms over others, have important effects on community structure. Still, the

functions of most plant chemical signals, and the genetic elements that orchestrate plant and animal responses to them, remain largely unknown.

Chemical communication in the rhizosphere between plants and between plants and other soil organisms is often mediated by root-secreted plant compounds. Plant roots are capable of secreting an impressive array of chemical signals. For example, *Arabidopsis thaliana* roots excrete approximately 289 compounds (Walker *et al.*, 2003), a number of which are phenolic compounds with antimicrobial activity that may ultimately deter pathogen infection (Bais *et al.*, 2005). Conversely, isoflavonoids present in the root exudates of legumes serve as chemoattractants of compatible rhizobia, facilitating beneficial symbiotic associations (Long, 1996). Similarly, rhizo-secreted sesquiterpenes increase a branching factor in mycorrhizal fungi, mediating positive plant–fungal interactions (Akiyama *et al.*, 2005). Several compounds secreted by roots also mediate parasitic plant–host interactions, inducing both germination and formation of specialized root structures (i.e. haustoria) in parasitic plants (Estabrook & Yoder, 1998; Yoder, 2001). Finally, some root exudates are phytotoxic and mediate negative plant–plant communication (i.e. allelopathy), inhibiting the growth, development, and survival of neighboring plants (Bertin *et al.*, 2003; Weir *et al.*, 2003).

Recent studies have shown that the European plant *Centaurea maculosa* (spotted knapweed), an invasive species in North America, rhizosecretes a phytotoxin (\pm)-catechin (Bais *et al.*, 2002, 2003), although the quantities of (\pm)-catechin produced and its potency as a phytotoxin under some conditions remain uncertain (Blair *et al.*, 2005). The molecular and biochemical mechanisms by which (\pm)-catechin induces plant growth inhibition and mortality have been investigated using the model plant *A. thaliana*. Application of minimum inhibitory concentrations (MICs) of (\pm)-catechin *in vitro* ($100 \mu\text{g mL}^{-1}$) to *A. thaliana* roots triggers a wave of reactive oxygen species (ROS) initiated at the root meristem, which leads to a Ca^{2+} signaling cascade and triggers genome-wide changes in gene expression, ultimately resulting in root death (Bais *et al.*, 2003). However, while earlier studies suggested that (\pm)-catechin concentrations in *C. maculosa* soils can be very high (Bais *et al.*, 2003; Perry *et al.*, 2005), more recent and extensive research indicates that soil (\pm)-catechin concentrations are most often quite low. In a study of soil (\pm)-catechin at 11 sites on a range of sampling dates, (\pm)-catechin was below the levels we could detect for almost all sites and sampling dates (L. Perry, unpublished). When (\pm)-catechin was present at concentrations we could detect, (\pm)-catechin concentrations in soil microsites within a single *C. maculosa* population varied from 0.14 to 2.18 mg g^{-1} (L. Perry, in preparation). Effects of low (\pm)-catechin concentrations on susceptible plants have not been examined.

Toxicological research suggests that many toxins can have entirely different effects on target organisms at low and at high

concentrations; in particular, low concentrations of toxins often result in improved growth and survival rather than growth inhibition or mortality, a phenomenon termed 'hormesis' (Calabrese & Baldwin, 2003a). While, historically, hormesis has been considered a paradox without general importance, recent reviews have indicated that hormesis is surprisingly common and have led to a greater appreciation of the potential importance of hormesis to issues in environmental pollution and medicine (Calabrese & Baldwin, 2003b; Calabrese, 2005). However, the potential for hormesis to play a role in chemical communication between plants and between plants and microbes in ecological systems has not been examined. Here we report that low concentrations of (\pm)-catechin, a root-exuded phytotoxin, result in chemical facilitation and induced pathogen defense responses in *A. thaliana* and we use *A. thaliana* mutants to elucidate the mechanisms behind this case of hormesis.

Materials and Methods

Plant material, bacterial culture and chemicals

Arabidopsis thaliana (L.) Heynath ecotype Columbia (Col-0) seeds were purchased from Lehle Seed Company (Roundrock, TX, USA). *Arabidopsis thaliana nabG* (salicylic acid hydroxylase) and *npr1-1* (nonexpressor of pathogenesis related protein 1-1) seeds were provided by Dr Xinnian Dong (Duke University, Durham, NC, USA). A culture of *Pseudomonas syringae* pv. *tomato* DC3000 was provided by Dr Frederick Ausubel (Department of Genetics, Harvard Medical School, Boston, MA, USA). Seeds of a transgenic line of *A. thaliana* carrying the pathogenesis-related protein 1 (*PR1*) gene promoter fused to a β -glucuronidase (GUS) reporter (Shapiro & Zhang, 2001) were obtained from Dr Allan Shapiro (University of Delaware, Newark, DE, USA). (\pm)-Catechin and all other chemicals were purchased from Sigma (St Louis, MO, USA) unless otherwise stated.

Germination and growing conditions

For all experiments, *A. thaliana* seeds were surface-sterilized in 2% sodium hypochlorite for 1 to 2 min and rinsed three times in sterile distilled water before germination. *A. thaliana* seeds were Col-0 unless otherwise stated. For *in vitro* experiments, seeds were plated on solidified Murashige and Skoog (MS; Murashige & Skoog, 1962) basal medium supplemented with 30 g l^{-1} sucrose and placed in a growth chamber (Percival Scientific Inc., Perry, IA, USA) for germination. For experiments in soil, seeds were planted in sterile peat pellets (Jiffy Co., Shippegan, New Brunswick, Canada), irrigated and placed in a growth chamber for germination. All experiments were conducted in a growth chamber at $25 \pm 2^\circ\text{C}$ with a day:night cycle of 16 : 8 h unless otherwise stated.

Effect of (\pm)-catechin on *A. thaliana* growth *in vitro*

Two weeks after sowing on solidified MS basal medium, *A. thaliana* plants were transferred onto solidified MS medium supplemented with different concentrations of (\pm)-catechin (0, 5, 10, 25, 50, 100, 400 and 800 $\mu\text{g ml}^{-1}$) dissolved in methanol and diluted with water. Each Petri dish contained five plants and there were three replicate dishes per (\pm)-catechin treatment. Plants were allowed to grow on this medium for 10 d and were then harvested, dried to a constant weight in a forced-air oven at 70°C for 24 h, and weighed. The control treatment received a volume of methanol (0.8% volume/volume (v/v)) equivalent to that present in the highest concentration of (\pm)-catechin. A series of controls containing 0.1, 0.25, 0.4, 0.5 and 0.8% (v/v) methanol were examined separately by addition of appropriate volumes of methanol to MS basal medium before pouring into Petri plates; the temperature of the medium was approx. 45–50°C at the time at which methanol was added.

Effect of (\pm)-catechin on *A. thaliana* growth in soil

Two weeks after sowing in peat pellets, the peat pellets with *A. thaliana* plants were placed in Magenta jars (Magenta Inc., Chicago, IL, USA) and irrigated with solutions of (\pm)-catechin such that the final concentrations of (\pm)-catechin were 0.0, 0.1, 1.0, 10 and 100 $\mu\text{g g}^{-1}$ wet weight with five replicate peat pellets per (\pm)-catechin treatment. All the treatments received an equal volume of methanol (500 μl). The plants were grown for 2 wk, after which the above-ground biomass was harvested, dried in a forced-air oven at 70°C for 48 h and weighed.

Effect of (\pm)-catechin on accumulation of ROS in *A. thaliana*

Three weeks after sowing on MS basal medium, *A. thaliana* plants were transferred onto MS basal medium supplemented with 0–25 $\mu\text{g ml}^{-1}$ (\pm)-catechin, using a 10 mg ml^{-1} (\pm)-catechin stock solution prepared in methanol. Each treatment had five replicate Petri dishes with five plants in each Petri dish. The control plants received a volume of methanol equivalent to that present in the highest (\pm)-catechin concentration. After 24 h the plants were removed and placed in a 3.5-cm-diameter Petri dish containing 0.2% nitro blue tetrazolium chloride (NBT) for 30 min. At the end of the incubation period the plants were destained in two changes of ethanol:acetic acid (70 : 30) solution and photographed. Changes in the root morphology and ROS in the deformed roots were observed after 7 d of treatment.

(\pm)-Catechin treatment of *A. thaliana* roots followed by leaf inoculation with *P. syringae* pv. *tomato* DC3000

Seedlings of *A. thaliana* Col-0, *nahG*, and *npr1-1* were grown on MS basal medium for 3 wk in Magenta jars. The plants were

then transferred onto MS basal medium supplemented with 0–25 $\mu\text{g ml}^{-1}$ (\pm)-catechin (a 10 mg ml^{-1} (\pm)-catechin stock solution prepared in methanol was used) for 2 d. Each treatment had 10 replicate plants. The leaves of these plants were inoculated with *P. syringae* pv. *tomato* DC3000 as described by Cui *et al.* (2002) and the inoculated plants were then transferred to a growth chamber set at a constant temperature of 30°C and a 16 : 8 h day:night cycle. The control plants received a volume of methanol equivalent to that present in the highest (\pm)-catechin concentration. After 2 and 3 d the area infected on each leaf was measured. The area was calculated by tracing the whole leaf and the portions of the leaf infected on graph paper, and the per cent area of the leaf infected was then calculated.

Induction of *PR1* expression by root treatment with (\pm)-catechin

Two weeks after sowing, plants of a transgenic line of *A. thaliana* carrying the *PR1* promoter fused to a *GUS* reporter were transferred to 12-well plates (Corning, NY, USA) containing liquid MS basal medium supplemented with 0, 10, 25 or 50 $\mu\text{g ml}^{-1}$ (\pm)-catechin, using a 10 mg ml^{-1} stock solution prepared in methanol. The control plants were transferred to MS medium supplemented with a volume of methanol equivalent to that present in the highest (\pm)-catechin concentration. Five plants from each treatment level were removed 24, 48 and 72 h after treatment, and stained for *GUS* expression using published methods (Jefferson, 1987). Briefly, the whole plant was placed in a staining solution consisting of 50 mM NaHPO_4 , pH 7.2, 0.5% Triton X-100, and 1 mM X-Gluc diluted from a 20 mM stock solution made up in dimethylformamide and incubated at 37°C overnight. At the end of the incubation period the chlorophyll was removed by incubation in several changes of 70% ethanol and the plants were photographed. For microscopic observation, pieces of leaf tissue were mounted on a glass slide and observed under an Olympus BX60 microscope equipped with COOLSNAP imaging software (MediaCybernetics, San Diego, CA, USA).

Effect of (\pm)-catechin on callose deposition, cell death and ROS in leaves of *A. thaliana*

Two weeks after sowing, *A. thaliana* plants were transferred onto Petri plates containing solidified MS medium containing 0–25 $\mu\text{g ml}^{-1}$ (\pm)-catechin (a 10 mg ml^{-1} (\pm)-catechin stock solution prepared in methanol was used). The control plants received a volume of methanol equivalent to that present in the highest (\pm)-catechin concentration. After 24 h, fully expanded leaves were excised and stained to visualize callose deposition following published methods (Stone *et al.*, 2000). Cell death was observed after staining with lacto-phenol blue (Dietrich *et al.*, 1994; Bowling *et al.*, 1997), while ROS localization in the leaves was visualized after nitroblue tetrazolium staining (Jabs *et al.*, 1996). Microscopic observation

was carried out using a fluorescence microscope (Olympus BX60) equipped with COOLSNAP imaging software (Media-Cybernetics). Leaves from at least five plants from each treatment were stained and microscopically observed.

Results and Discussion

Low concentrations of (\pm)-catechin induce growth in *A. thaliana*

We previously reported that the MIC of ($-$)-catechin for *A. thaliana* is $50 \mu\text{g ml}^{-1}$ under *in vitro* conditions in liquid MS medium (Bais *et al.*, 2002). However, we found that when (\pm)-catechin was applied to plants in solid MS medium, the MIC for *A. thaliana* was $400 \mu\text{g ml}^{-1}$ (Fig. 1a). Moreover, treatment with lower concentrations of (\pm)-catechin in solid medium ($25 \mu\text{g ml}^{-1}$) promoted the growth of *A. thaliana* seedlings, and this growth promotion was concentration dependent. *A. thaliana* growth was greatest (twice that of control plants) for plants treated with $25 \mu\text{g ml}^{-1}$ (\pm)-catechin (Fig. 1a). The growth-promoting activity diminished at a concentration of $50 \mu\text{g ml}^{-1}$ (Fig. 1a). Different concentrations of methanol used as controls did not affect the growth of *A. thaliana* seedlings (data not shown). Similar positive effects of (\pm)-catechin on *A. thaliana* growth were observed under soil conditions (Fig. 1b,c). However, the (\pm)-catechin concentration required for maximum growth in soil was $1.0 \mu\text{g g}^{-1}$. With $1.0 \mu\text{g g}^{-1}$ of (\pm)-catechin in soil, shoot biomass more than doubled; increases in shoot dry matter were also observed with $0.1 \mu\text{g g}^{-1}$ soil but were less pronounced. Higher (\pm)-catechin concentrations ($100 \mu\text{g g}^{-1}$) resulted in plant mortality, in accord with earlier studies (Bais *et al.*, 2003).

Several toxins, including antibiotics and anthropogenic pesticides, are known to improve the growth of target organisms when applied at sublethal concentrations (i.e. hormesis) (Calabrese & Baldwin, 2001, 2003a,b). As hormesis has been observed only under stringent experimental conditions, the extent to which hormesis occurs in nature is unknown. The results of our experiments with *A. thaliana* in soil suggest that low (\pm)-catechin concentrations in *C. maculosa* soils might sometimes result in hormesis in natural plant communities. (\pm)-Catechin concentrations in *C. maculosa* soils in North America are often lower than we can detect, suggesting that when (\pm)-catechin is present it is at concentrations much lower than MIC levels for susceptible plants (L. Perry, unpublished). The improved growth of *A. thaliana* (Fig. 1a) when exposed to very low levels of (\pm)-catechin (e.g. $1.0 \mu\text{g g}^{-1}$) suggests that the neighbors of *C. maculosa* may sometimes benefit from the presence of this compound. The effect of low concentrations of (\pm)-catechin on the growth of *Achillea millefolium* and *Lotus corniculatus*, which are native to Europe, was tested. Interestingly, (\pm)-catechin promoted the growth of both plant species although the effective concentration varied between the plant species (data not shown).

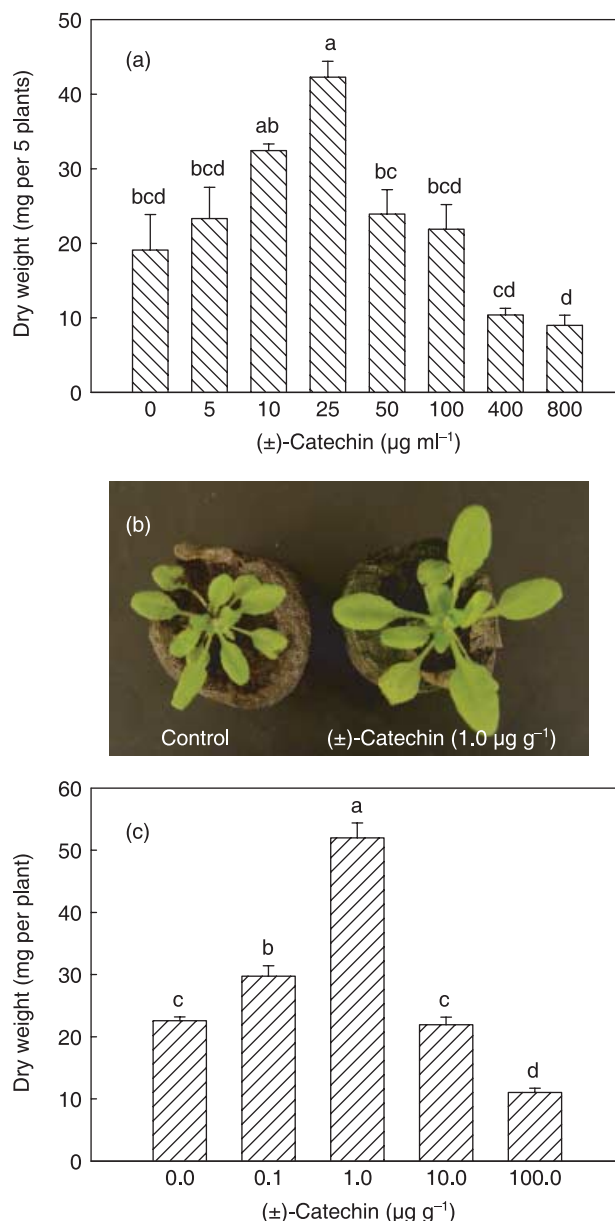
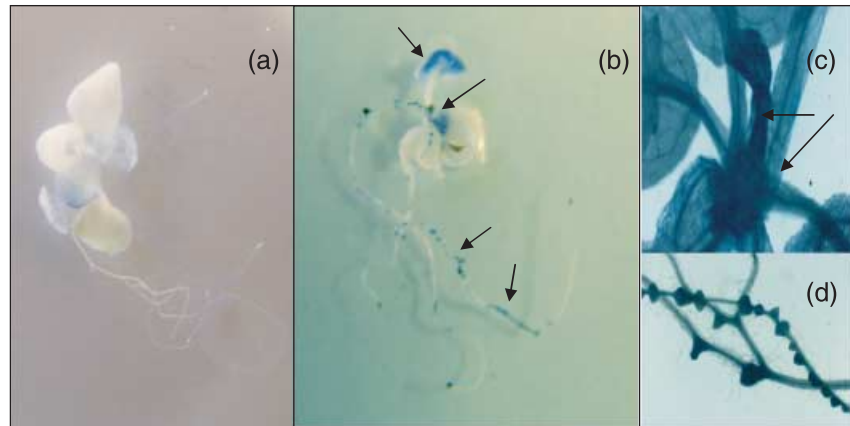


Fig. 1 Root treatment with (\pm)-catechin enhanced growth and biomass accumulation of *Arabidopsis thaliana* Columbia (Col-0). (a) Addition of $25 \mu\text{g ml}^{-1}$ (\pm)-catechin significantly induced growth of *A. thaliana*, more than doubling mean *A. thaliana* biomass (analysis of variance (ANOVA), $F_{7,16} = 13.68$, $P < 0.0001$) under *in vitro* conditions. (b) Growth facilitation in *A. thaliana* Col-0 produced by addition of (\pm)-catechin to soil; maximum growth facilitation occurred at $1.0 \mu\text{g g}^{-1}$ soil (\pm)-catechin treatment. (c) Addition of low (\pm)-catechin concentrations (0.1 and $1.0 \mu\text{g g}^{-1}$) to soil significantly enhanced the growth and dry matter accumulation of *A. thaliana* Col-0 plants (ANOVA, $F_{4,20} = 105.30$, $P < 0.0001$). (Error bars are 1 standard error of the mean. Different letters indicate significantly different means (Tukey honestly significant differences (HSD), $P < 0.05$.)

Fig. 2 Treatment of *Arabidopsis thaliana* with sub-minimum inhibitory concentration (MIC) levels of (\pm)-catechin induced production of reactive oxygen species (ROS) in root and shoot meristems. (a, b) Ten-d-old *A. thaliana* plants were transferred onto solid Murashige and Skoog medium supplemented with sub-MIC levels of (\pm)-catechin ($5\text{--}25\ \mu\text{g ml}^{-1}$) for 24 h and then stained for ROS with nitroblue tetrazolium and photographed: (a) control; (b) treated. (c) A close-up view of the shoot meristem showing ROS accumulation. Arrows in (b) and (c) indicate ROS localization. (d) Roots of *A. thaliana* exposed to (\pm)-catechin developed meristematic outgrowths that showed accumulation of ROS.



(\pm)-Catechin treatment induced ROS accumulation in *A. thaliana*

The mechanisms by which (\pm)-catechin enhances plant growth are not clear, but may involve chemical effects on cell development in meristematic tissue. Loosening of the cell walls is an essential step in meristem growth. Cell wall loosening can occur by two processes: enzymatic reactions involving expansins, endoglucanases or xyloglucan endotransglycosylase (McQueen-Mason, 1995; Cosgrove, 1999), or a nonenzymatic process mediated by ROS (Miller, 1986; Fry, 1998; Schweikert *et al.*, 2000). Recently, Rodriguez *et al.* (2002) demonstrated that ROS generation in the expansion zone of maize (*Zea mays*) leaf blades is essential for leaf growth. Previous studies have shown that ROS can be generated by several mechanisms including action of the antioxidant ascorbate (Fry, 1998). Additionally, it has been shown that NADPH-dependent ROS is involved in root hair elongation and development (Foreman *et al.*, 2003). In the present study the control plants accumulated basal levels of ROS (Fig. 2a), while in the treated plants we observed enhanced accumulation of ROS in the shoot and root meristems of (\pm)-catechin-treated plants (Fig. 2b–d), which might account at least in part for improved growth. ROS generation has also been observed in the roots of *A. thaliana* treated with high concentrations of (\pm)-catechin ($100\ \mu\text{g ml}^{-1}$), ultimately resulting in plant mortality (Bais *et al.*, 2003). Low (\pm)-catechin concentrations may elicit moderate levels of ROS which promote growth while higher (\pm)-catechin concentrations elicit high levels of ROS, leading to cell death.

(\pm)-Catechin treatment of *A. thaliana* roots induces shoot resistance to *P. syringae* pv. *tomato* DC3000 infection

Treatment of *A. thaliana* with (\pm)-catechin induces up-regulation of several genes that are related to pathogen resistance, including pathogenesis-related proteins and antioxidant

response genes (Bais *et al.*, 2003). Therefore, we predicted that (\pm)-catechin secreted by *C. maculosa* roots may induce the expression of *PR1* genes of neighboring plants, resulting in the induction of the defense response of the neighbors against pathogens. To test this hypothesis, we transferred 20-d-old *A. thaliana* plants grown on MS basal medium onto MS basal medium supplemented with lower-than-MIC concentrations of (\pm)-catechin ($5\text{--}25\ \mu\text{g ml}^{-1}$) for 24 and 48 h. Leaves of these plants were then inoculated with *P. syringae* pv. *tomato* DC3000, a virulent pathogen of *A. thaliana* (Jakob *et al.*, 2002). *A. thaliana* plants exposed to (\pm)-catechin developed restricted lesions only at the site of inoculation, while the control plants exhibited widespread *P. syringae* pv. *tomato* DC3000 infections, resulting in the complete collapse of inoculated leaves (Table 1).

Root treatment of *A. thaliana* with lower-than-MIC levels of (\pm)-catechin induced the expression of *PR1* in the leaves

Organic compounds such as salicylic acid (SA), benzothiadiazole and isonicotinic acid are known to induce plant resistance to a number of pathogenic microbes in a process known as systemic acquired resistance (SAR) (Shah, 2003). The *PR1* gene has been implicated at least in part in the induction of disease resistance in plants (Delaney *et al.*, 1994; Van Loon, 1997; Shah, 2003), and we therefore tested whether the exposure of *A. thaliana* roots to (\pm)-catechin caused an up-regulation of *PR1*. To test whether exposure of *A. thaliana* roots to (\pm)-catechin elicits disease resistance responses through induction of *PR1* genes in above-ground tissue, we used transgenic *A. thaliana* plants in which the *PR1* promoter is fused to a *GUS* reporter gene (Shapiro & Zhang, 2001). Root treatment of *A. thaliana* with (\pm)-catechin at lower-than-MIC levels ($5\text{--}25\ \mu\text{g ml}^{-1}$) resulted in induction of the *PR1* gene in leaves; at 24 h *PR1* expression was observed in the petioles and parts of the leaf lamina, while at 72 h *PR1* expression was observed throughout the leaves and control

Table 1 Effect of pretreatment of *Arabidopsis thaliana* with (\pm)-catechin on the development of *Pseudomonas syringae* pv. *tomato* DC3000 on leaves

Genotype	% leaf area infected			
	48 h		72 h	
	Control	(\pm)-Catechin	Control	(\pm)-Catechin
Col-0	36.0 \pm 12.9	9.1 \pm 7.4	55.0 \pm 13.2	16.0 \pm 8.0
<i>nahG</i>	52.0 \pm 8.3	45.0 \pm 13.2	78.0 \pm 14.0	75.0 \pm 17.6
<i>npr1-1</i>	57.0 \pm 20.9	48.0 \pm 18.2	89.0 \pm 11.9	80.0 \pm 20.9

Values are means \pm 1 standard error.

Col-0, Columbia; *nahG*, salicylic acid hydroxylase; *npr1-1*, nonexpressor of pathogenesis related protein 1-1.

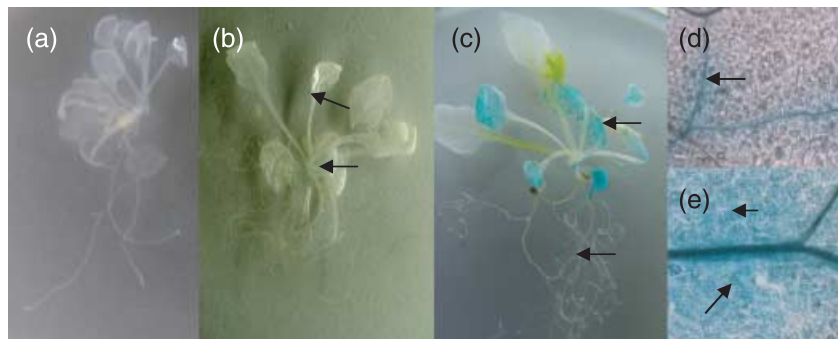


Fig. 3 Root treatment with 10–25 $\mu\text{g ml}^{-1}$ (\pm)-catechin induced pathogenesis-related protein 1 (*PR1*) gene expression. A transgenic line carrying *PR1::GUS* was grown in Murashige and Skoog (MS) basal medium for 2 wk and transferred to MS basal medium supplemented with 10–25 $\mu\text{g ml}^{-1}$ (\pm)-catechin and stained for *GUS* expression. (a) Control plants did not show inductions of *GUS* expression after 72 h. (b) After 24 h, patches of expression were seen in the petiole and the lamina (arrows). (c) After 72 h, intense expression was observed in the leaves and parts of the roots (arrows). (d) *PR1* expression was initially localized in the midrib and the veins, and (e) later spread to the entire lamina.

plants did not show *GUS* expression (Fig. 3a–e). The leaves at the top of the rosette exhibited the maximum induction of *PR1* as observed by intense *GUS* staining (Fig. 3c). Interestingly, *PR1* expression was also observed in sections of the root system (Fig. 3c, arrow) suggesting that (\pm)-catechin treatment may also protect plants against root pathogens. At a mechanistic level, our results suggest that (\pm)-catechin may be detected by plant roots as a potential defense-inducing factor similar to those produced by avirulent pathogens leading to plant resistance.

(\pm)-Catechin induced resistance to *P. syringae* pv. *tomato* DC3000 is salicylic acid and NPR1 dependent

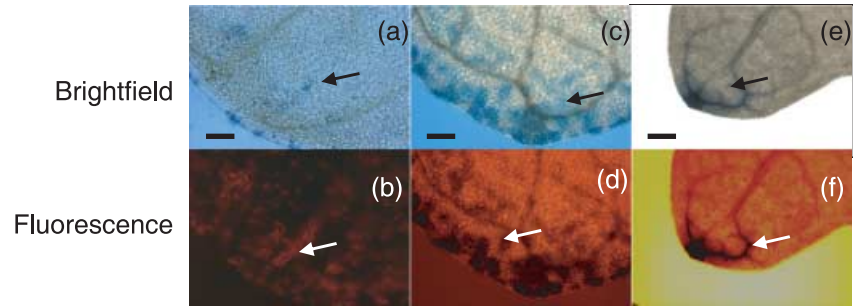
SAR induction in plants is mediated by SA (Shah, 2003). However, recent studies have shown that some forms of induced resistance mediated by plant-growth promoting rhizobacteria are SA independent (Penninckx *et al.*, 1996; Thomma *et al.*, 1998; Pieterse & van Loon, 1999; Ryu *et al.*, 2004). To test whether (\pm)-catechin-induced defense responses are mediated by SA, we tested two *A. thaliana* genotypes altered in disease resistance, *nahG* and *npr1-1*. *nahG* is a transgenic line that lacks the ability to accumulate SA, and *npr1-1* is a

mutant impaired in a transcriptional regulator that acts downstream of SA accumulation. Both plants have demonstrated reduced *PR1* gene expression (Gaffney *et al.*, 1993; Delaney *et al.*, 1994; Cao *et al.*, 1994). We preconditioned the *A. thaliana* mutants with 10 $\mu\text{g ml}^{-1}$ (\pm)-catechin for 24 h and then inoculated the leaves with *P. syringae* pv. *tomato* DC3000. As in our previous experiments, wild-type *A. thaliana* pretreated with (\pm)-catechin was relatively resistant to *P. syringae* pv. *tomato* DC3000, with infections restricted to the site of inoculation (Table 1). However (\pm)-catechin pretreatment of the defense response mutants *nahG* and *npr1-1* did not increase resistance to *P. syringae* pv. *tomato* DC3000 infection. Instead, infections were similar in control and (\pm)-catechin pretreated plants. These results suggest that the mechanism by which (\pm)-catechin induces disease resistance in *A. thaliana* is mediated by the SA/NPR1-dependent pathway.

Root treatment of *A. thaliana* with (\pm)-catechin affects callose deposition, cell death and ROS accumulation in the leaves

To further investigate the commonalities between the response to phytotoxins and more traditional plant defense

Fig. 4 Root treatment of *Arabidopsis thaliana* with (\pm)-catechin induced pathogenesis-related responses in the leaves. (a) A leaf stained with aniline blue revealing callose deposition (arrow); (b) epifluorescence of (a). (c) A leaf stained with lactophenol-trypan blue revealing cell death (arrow); (d) epifluorescence of (c). (e) A leaf stained with nitroblue tetrazolium revealing reactive oxygen species (ROS) (arrow); (f) epifluorescence of (e).



responses, we documented all traditional physiological plant defense responses we observed. Treatment of *A. thaliana* roots with lower-than-MIC levels of (\pm)-catechin induced several pathogen-related responses. Figure 4(a) and (b) show significant deposition of callose in the leaves. A closer look at the site of deposition of callose reveals large amounts of callose in the cell walls. Similarly, root treatment of *A. thaliana* with (\pm)-catechin induced cell death in the leaves (Fig. 4c,d), which was localized mainly on the leaf perimeter. Further (\pm)-catechin treatment induced ROS generation (Fig. 4e,f). Toxins produced by a number of pathogens, such as *Fusarium moliniforme* and *Alternaria alternata* f sp. *lycopersici*, induce a similar reaction in the leaves of treated plants (Gilchrist, 1998; Stone *et al.*, 2000). *A. thaliana* responses to (\pm)-catechin treatment suggest that (\pm)-catechin may be perceived as a pathogen-derived signal by the roots of *A. thaliana*.

Conclusions

The concept of chemical facilitation has a long history, originating over a century ago in research by Schulz (1887), who noted that many chemicals stimulate growth and respiration of yeast at low doses but are inhibitory at higher concentrations. This concept of a generalized low-dose stimulation/high-dose inhibition has gradually accumulated support from similar observations with other chemicals and is known as the Arndt-Schulz law (Calabrese & Baldwin, 2001). Despite the widespread recognition of apparent hormetic or chemical facilitation effects, researchers are still puzzled by the basic mechanisms of the opposing effects, such as the increased and reduced biomasses that occurred at different (\pm)-catechin concentrations in our experiments. We have put forward one possible explanation for enhanced growth caused by low levels of (\pm)-catechin in the *A. thaliana* model, that low concentrations of (\pm)-catechin elicit optimum ROS in the meristem, which in turn triggers cell division and cell expansion. Further, we have demonstrated that low (\pm)-catechin concentrations increase pathogen resistance by inducing up-regulation of pathogenesis-related genes such as *PR1* via the SA/NPR1-dependent pathway. However, it should be noted that elevated concentrations of SA could be toxic to plants (Bowling *et al.*, 1997; Petersen *et al.*, 2000).

Hormesis might include biological phenomena other than increased growth such as mild oxidative stress (Radak *et al.*, 2005), and in the present study we have found that hormesis responses could induce resistance to pathogens. If such interactions between below-ground interspecific plant signals and above-ground growth and responses to pathogens occur in natural settings, they could influence plant community structure in a number of ways, including alteration of resource uptake and reproduction, and indirect effects of pathogens on plant competition. The importance of such linkages between above-ground and below-ground systems for community productivity and diversity has only recently been appreciated (Wardle *et al.*, 2004), and the role of chemical signals in forging those linkages is only beginning to be understood. The potential for plant compounds to serve as toxins against susceptible plants at higher concentrations, but as signals for facilitation of plant growth and disease resistance at lower concentrations, highlights the importance of considering multiple effects above-ground and below-ground, including hormesis, when examining the biological and ecological role of plant secondary metabolites. Future studies on the differential induction of genome-wide changes elicited by MIC and sub-MIC levels of (\pm)-catechin may reveal the molecular mechanisms that underlie this intriguing phenomenon.

Acknowledgements

This research was supported by grants from the National Science Foundation (IBN 0335203) and the Department of Defense SERDP (CS1388) to JMV.

References

- Akiyama K, Matsuzaki K, Hayashi H. 2005. Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. *Nature* 435: 824–827.
- Bais HP, Prithiviraj B, Jha AK, Ausubel FM, Vivanco JM. 2005. Mediation of pathogen resistance by exudation of antimicrobials from roots. *Nature* 434: 217–221.
- Bais HP, Vepachedu R, Gilroy S, Callaway RM, Vivanco JM. 2003. Allelopathy and exotic plant invasion: from molecules and genes to species interactions. *Science* 301: 1377–1380.
- Bais HP, Walker TS, Stermitz FR, Hufbauer RA, Vivanco JM. 2002. Enantiomeric-dependent phytotoxic and antimicrobial activity of (+/-)-

- catechin. A rhizosecreted racemic mixture from spotted knapweed. *Plant Physiology* 128: 1173–1179.
- Baker B, Zambryski P, Staskawicz B, Dinesh-Kumar SP. 1997. Signaling in plant–microbe interactions. *Science* 276: 726–733.
- Bertin C, Yang XH, Weston LA. 2003. The role of root exudates and allelochemicals in the rhizosphere. *Plant and Soil* 256: 67–83.
- Blair AC, Hanson BG, Brunk GR, Marrs RA, Westra P, Nissen SJ, Hufbauer RA. 2005. New techniques and findings in the study of a candidate allelochemical implicated in invasion success. *Ecology Letters* 8: 1039–1047.
- Bowling SA, Clarke JD, Liu Y, Klessig DF, Dong X. 1997. The *cpr5* mutant of *Arabidopsis* expresses both NPR1-dependent and NPR1-independent resistance. *Plant Cell* 9: 1573–1584.
- Bruin J, Dicke M, Sabelis MW. 1992. Plants are better protected against spider-mites after exposure to volatiles from infested conspecifics. *Experientia (Basel)* 48: 525–529.
- Calabrese EJ. 2005. Historical blunders: How toxicology got the dose–response relationship half right. *Cellular and Molecular Biology* 51: 643–654.
- Calabrese EJ, Baldwin LA. 2001. U-shaped dose–responses in biology, toxicology, and public health. *Annual Review of Public Health* 22: 15–33.
- Calabrese EJ, Baldwin LA. 2003a. Hormesis: the dose–response revolution. *Annual Review of Pharmacology and Toxicology* 43: 175–197.
- Calabrese EJ, Baldwin LA. 2003b. Toxicology rethinks its central belief. *Nature* 421: 691–692.
- Callaway RM. 1995. Positive interactions among plants. *Botanical Review* 61: 306–349.
- Cao H, Bowling SA, Gordon S, Dong X. 1994. Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. *Plant Cell* 6: 1583–1592.
- Cosgrove DJ. 1999. Enzymes and other agents that enhance cell wall extensibility. *Annual Review of Plant Physiology and Plant Molecular Biology* 50: 391–417.
- Cui J, Jander G, Racki LR, Kim PD, Pierce NE, Ausubel FM. 2002. Signals involved in *Arabidopsis* resistance to *Trichoplusia ni* caterpillars induced by virulent and avirulent strains of the phytopathogen *Pseudomonas syringae*. *Plant Physiology* 129: 551–564.
- Delaney T, Uknes S, Vernooij B, Friedrich L, Weymann K, Negrotto D, Gaffney T, Gut-Rella M, Kessman H, Ward E. 1994. A central role of salicylic acid in plant disease resistance. *Science* 266: 1247–1250.
- Dicke M, De Boer JG, Hofte M, Rocha-Granados MC. 2003. Mixed blends of herbivore-induced plant volatiles and foraging success of carnivorous arthropods. *Oikos* 101: 38–48.
- Dicke M, Van Beek TA, Posthumus MA, Ben Dom N, Van Bokhoven H, De Groot AE. 1990. Isolation and identification of volatile kairomone that affects acarine predator–prey interactions involvement of host plant in its production. *Journal of Chemical Ecology* 16: 381–396.
- Dietrich RA, Delaney TP, Uknes SJ, Ward ER, Ryals JA, Dangel JL. 1994. *Arabidopsis* mutants simulating disease resistance response. *Cell* 77: 565–577.
- Estabrook EM, Yoder JI. 1998. Plant–plant communications: rhizosphere signaling between parasitic angiosperms and their hosts. *Plant Physiology* 116: 1–7.
- Foreman J, Demidchik V, Bothwell JH, Mylona P, Miedema H, Torres MA, Linstead P, Costa S, Brownlee C, Jones JD, Davies JM, Dolan L. 2003. Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature* 422: 442–446.
- Fry SC. 1998. Oxidative scission in plant cell wall polysaccharides by ascorbate-induced hydroxyl radicals. *Biochemical Journal* 332: 507–511.
- Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye G, Uknes S, Ward E, Kessmann H, Ryals J. 1993. Requirement of salicylic acid for the induction of systemic acquired resistance. *Science* 261: 754–756.
- Gilchrist DG. 1998. Programmed cell death in plant disease: The purpose and promise of cellular suicide. *Annual Review of Phytopathology* 36: 393–414.
- Hirsch AM, Bauer WD, Bird DM, Cullimore J, Tyler B, Yoder JI. 2003. Molecular signals and receptors: controlling rhizosphere interactions between plants and other organisms. *Ecology* 84: 858–868.
- Jabs T, Dietrich RA, Dangel JL. 1996. Initiation of runaway cell death in an *Arabidopsis* mutant by extracellular superoxide. *Science* 273: 1853–1856.
- Jakob K, Goss EM, Van Araki HT, Kreitman M, Bergelson J. 2002. *Pseudomonas viridiflava* and *P. syringae* – natural pathogens of *Arabidopsis thaliana*. *Molecular Plant–Microbe Interactions* 15: 1195–1203.
- Jefferson RA. 1987. Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Molecular Biology Reporter* 5: 387–405.
- Kessler A, Baldwin IT. 2001. Defensive function of herbivore-induced plant volatile emissions in nature. *Science* 291: 2141–2144.
- Long SR. 1996. Rhizobium symbiosis: nod factors in perspective. *Plant Cell* 8: 1885–1898.
- McQueen-Mason SJ. 1995. Expansins and cell wall expansion. *Journal of Experimental Botany* 46: 1639–1650.
- Miller AR. 1986. Oxidation of cell wall polysaccharides by hydrogen peroxide: a potential mechanism for cell wall breakdown in plants. *Biochemistry and Biophysics Research Communications* 141: 238–244.
- Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassay with tissue culture. *Physiologia Plantarum* 15: 473–476.
- Penninckx IAMA, Eggermont K, Terras FRG, Thomma BPHJ, De Samblanx GW, Buchala A, Métraux J-P, Manners JM, Broekaert WF. 1996. Pathogen-induced systemic activation of a plant defensin gene in *Arabidopsis* follows a salicylic acid–independent pathway. *Plant Cell* 8: 2309–2323.
- Perry LG, Thelen GC, Ridenour WM, Weir TL, Callaway RM, Paschke MW, Vivanco JM. 2005. Dual role for an allelochemical: (±)-catechin from *Centaurea maculosa* root exudates regulates conspecific seedling establishment. *Journal of Ecology* 93: 1126–1135.
- Petersen M, Brodersen P, Naested H, Andreasson E, Lindhart U, Johansen B, Nielsen HB, Lacy M, Austin MJ, Parker JE, Sharma SB, Klessig DF, Martienssen R, Mattsson O, Jensen AB, Mundy J. 2000. *Arabidopsis* map kinase 4 negatively regulates systemic acquired resistance. *Cell* 103: 1111–1120.
- Pieterse CMJ, van Loon LC. 1999. Salicylic acid-independent plant defense pathways. *Trends in Plant Sciences* 4: 52–58.
- Radak Z, Chung HY, Goto S. 2005. Exercise and hormesis: oxidative stress-related adaptation for successful aging. *Biogerontology* 6: 71–75.
- Rodriguez AA, Grunberg KA, Taleisnik EL. 2002. Reactive oxygen species in the elongation zone of maize leaves are necessary for leaf extension. *Plant Physiology* 129: 1627–1632.
- Ryu CM, Farag MA, Hu CH, Reddy MS, Kloepper JW, Pare PW. 2004. Bacterial volatiles induce systemic resistance in *Arabidopsis*. *Plant Physiology* 134: 1017–1026.
- Schulz H. 1887. Zur Lehre von der Arzneiwirkung. *Virchows Archiv für Pathologische Anatomie und Physiologie und für Klinische Medizin* 108: 423–445.
- Schweikert C, Liszky A, Schopfer P. 2000. Scission of polysaccharides by peroxidase-generated hydroxyl radicals. *Phytochemistry* 53: 565–570.
- Shah J. 2003. Salicylic acid loop in plant defense. *Current Opinion in Plant Biology* 6: 365–371.
- Shapiro AD, Zhang C. 2001. The role of *ndr1* in avirulence gene-directed signaling and control of programmed cell death in *Arabidopsis*. *Plant Physiology* 127: 1089–1101.
- Shulaev V, Silverman P, Raskin I. 1997. Airborne signaling by methyl salicylate in plant pathogen resistance. *Nature* 385: 718–721.
- Stoll P, Weiner J. 2000. A neighborhood view of interactions among individual plants. In: Stracke S, Kistner C, Yoshida S, Mulder L, Sato S, Kaneko T, Tabata S, Sandal N, Dieckmann R, Law R, Metz JAJ, eds. 1994. *The geometry of ecological interactions*. Cambridge, UK: Cambridge University Press, 11–28.

- Stone JM, Heard JE, Asai T, Ausubel FM. 2000. Simulation of fungal-mediated cell death by fumonisin B1 and selection of fumonisin B1-resistant (*fbr*) *Arabidopsis* mutants. *Plant Cell* 12: 1811–1822.
- Thomma BPHJ, Eggermont K, Penninckx IAMA, Mauch-Mani B, Vogelsang R, Cammue BPA, Broekaert WF. 1998. Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. *Proceedings of the National Academy of Sciences, USA* 95: 15107–15111.
- Van Loon LC. 1997. Induced resistance in plants and the role of pathogenesis-related proteins. *European Journal of Plant Pathology* 103: 753–765.
- Walker TS, Bais HP, Halligen KM, Stermitz FR, Vivanco JM. 2003. Metabolic profiling of non-polar compounds in root exudates of *Arabidopsis thaliana* *in vitro*; study of dynamic interface for the comprehensive characterization of rhizospheric interactions. *Journal of Agricultural and Food Chemistry* 51: 2548–2554.
- Wardle DA, Bardgett RD, Klironomos JN, Setälä H, van der Putten WH, Wall DH. 2004. Ecological linkages between aboveground and belowground biota. *Science* 304: 1629–1633.
- Weir TL, Bais HP, Vivanco JM. 2003. Intraspecific and interspecific interactions mediated by a phytotoxin, (–)-catechin, secreted by the roots of *Centaurea maculosa* (spotted knapweed). *Journal of Chemical Ecology* 29: 2397–2412.
- Yoder JL. 2001. Host plant recognition by parasitic Scrophulariaceae. *Current Opinion in Plant Biology* 4: 359–365.