

# A selective, sensitive, and rapid in-field assay for soil catechin, an allelochemical of *Centaurea maculosa*

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## Abstract

*Centaurea maculosa* is a plant species native to Eurasia and invasive in many regions of North America that secretes a phytotoxin from its roots, (+/–)-catechin, which is thought to serve an allelopathic role by reducing fitness of neighboring species. However, catechin is a relatively unstable compound, is often found at relatively low concentrations, interacts with soil cations to form insoluble complexes and/or degradation products, and exhibits extremely variable accumulation patterns in the soil. These factors, coupled with a lack of knowledge of the regulation of catechin production by *C. maculosa*, confound our understanding of the importance of catechin as an allelochemical. The time and cost of current sampling procedures effectively limit the observations made on catechin accumulation patterns, without which it is extremely difficult to conclusively attribute allelopathic significance to this compound. Thus, a highly sensitive, fast, inexpensive and reliable method for soil catechin analysis is needed. We report a novel method that utilizes the colorimetric reagent dimethylaminocinnamaldehyde (DMACA) in an acidic ethanol solution for detection of soil catechin. This method is selective and extremely sensitive and can be used in the field for qualitative, but not quantitative, analysis. This assay will allow for a greater understanding of the role of catechin as an allelochemical.

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## 1. Introduction

Numerous mechanisms for successful invasion by exotic plant species in novel habitats have been suggested (Shea and Chesson, 2002). One conjecture for the success of invasive species posits that plants that are introduced from a distant region possess phytochemicals that are novel, and therefore more detrimental, to pathogens, parasites, or competitors in the introduced range. The novelty of these weapons allows for successful establishment and ultimately range expansion and invasive behavior; this is known as the ‘novel weapons’ hypothesis (Callaway and Ridenour,

2004). This hypothesis is based in part on results suggesting that the European species *Centaurea maculosa* has gained a competitive advantage in North America partly through the use of the novel weapon (+/–)-catechin (Bais et al., 2003).

(+/–)-Catechin is thought to serve an allelochemical function, and this example has been considered one of the strongest for allelopathic behavior in plants (Fitter, 2003). *C. maculosa* root exudates have been found to possess phytotoxic properties, these exudates have been found by chemical analysis to contain catechin, and catechin supplied exogenously was found to inhibit growth and germination at concentrations reported to be exuded from *C. maculosa* under lab and field conditions (Bais et al., 2003). However, there is a great deal of variation in the levels of catechin recovered as root exudate from both lab and field studies, with some groups reporting only trace

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levels while others report milligram quantities per gram of soil (Bais et al., 2003; Blair et al., 2005, 2006; Perry et al., 2005; Thelen et al., 2005). Further, a recent study found that soil catechin concentrations can vary from very high to absent from 1 month to the next (Perry et al., 2007). The interpretation of these results are complicated by the fact that catechin is a relatively unstable compound, is often found at relatively low concentrations, interacts with soil cations to form insoluble complexes and/or degradation products, and exhibits extremely variable accumulation patterns. This variation in soil catechin concentrations suggests that frequent measurements at multiple sites, monitoring at daily or even hourly intervals, may be required to understand catechin dynamics in *C. maculosa* soils, and thus the role of catechin in *C. maculosa* invasion. However, such a large study would be difficult given the time and cost of soil extraction and analysis by current methods. A faster, less expensive method for soil catechin detection is needed.

Catechins (catechin and its diastereomer, epicatechin) are products of the flavonoid pathway (Tanner et al., 2003; Xie et al., 2003, 2004) and can be either end-products that accumulate under certain conditions or can serve as building blocks for condensed tannins, which are polymers of catechins (Porter, 1989). Condensed tannins have drawn research interest due to physiochemical properties that make them relevant to human health, plant defense, and agriculture (Dixon et al., 2005; Xie and Dixon, 2005). However, condensed tannins are chemically complex and difficult to structurally characterize. These properties necessitated a rapid assay for their presence, a need met by several colorimetric reagents, including the compound dimethylaminocinnamaldehyde (DMACA) (Porter, 1989). This compound is yellow-pigmented, but in the presence of alcohol solvents, strong mineral acid, and condensed tannin or related compounds, binds the tannin and becomes a vivid blue stain. These properties have allowed it to be used for histology (Abrahams et al., 2002; Feucht and Schmid, 1983; Gutmann, 1993), chemical analysis (Nagel and Glories, 1991; Qureshi et al., 1981; Solich et al., 1996; Treutter, 1989), and structural characterization (Treutter et al., 1994). DMACA recognizes the *meta*-orientated hydroxyls on the A ring, which imparts specificity (McMurrugh and McDowell, 1978; Treutter, 1989), and the vivid blue color with maximal absorbance in the long wavelength region of the visible spectrum makes the assay highly sensitive. These properties make it an attractive candidate for studying soil catechin levels in studies of *C. maculosa* allelopathy and the role of catechin in invasion.

This report describes a novel assay that employs DMACA for qualitative and semi-quantitative analysis of soil catechin. The method is selective, sensitive, and extremely fast, and can be conducted safely under field conditions. This should allow for more samples to be analyzed, leading to a greater understanding of the regulation of catechin accumulation in soil under field

conditions and ultimately for a greater understanding of the importance of catechin as an allelochemical.

## 2. Materials and methods

### 2.1. Assay development

Ethanol was obtained as absolute ethanol, but no attempts were made to remove trace water; thus there is likely to be ~5% water present as an azeotrope. DMACA was obtained from Sigma (# 49825). HCl was fuming (~37%) and obtained from Sigma (# 84436). All reactions were prepared as 200  $\mu$ L total volume containing 160  $\mu$ L of HCl solution, 20  $\mu$ L catechin solution (or soil extract), and 20  $\mu$ L DMACA solution. All solutions were prepared in ethanol unless otherwise noted. HCl solutions were prepared for 1, 2, 5, and 10% v/v. Catechin solutions were prepared as a 1000  $\mu$ g/mL solution, and serially diluted to 100, 10, 1, and 0.1  $\mu$ g/mL stocks. These solutions were diluted 10 times for assays, resulting in final concentrations of 100, 10, 1.0, 0.1, and 0.01  $\mu$ g/mL catechin. DMACA was prepared as a 4.0 mg/mL solution and serially diluted to 2.0, 1.0, 0.5, and 0.25 mg/mL. These values represent concentrations before 10-fold dilution into the assay solution. All assays were performed in 96-well plates, and the absorbance at 600 nm was used for quantification. Plates were read using a OspysMR plate reader (Dynex Technologies) fitted with a 600 nm filter. All reactions were set up with the 96-well plate resting on ice, to minimize variation in incubation start time and evaporative concentration of reagents.

### 2.2. Field samples

Field collected soil extracts from a previous study were analyzed for catechin content by the DMACA assay to validate the method. Soils were sampled from multiple locations (Table 1), and ~1.3 g soil samples were extracted in 10 mL methanol immediately upon collection, centrifuged and the supernatants concentrated to 0.4 mL (Perry et al., 2007). Each sample was initially diluted 10 times to obtain absorbance values in the linear range of the DMACA assay. 20  $\mu$ L of each diluted sample was added to a well containing 160  $\mu$ L of 1% HCl in ethanol and

Table 1  
Location of soils tested for DMACA false-positive readings

Site	Location	Latitude	Longitude
Canyon Ferry	MT, USA	46.30745°N	111.65037°W
Nelson Gulch 1	MT, USA	46.57040°N	112.14981°W
Clearwater	MT, USA	47.00726°N	113.37319°W
Mt. Sentinel	MT, USA	46.84102°N	113.98251°W
Big Creek	MT, USA	46.45580°N	114.18236°W
Petty Mountain	MT, USA	46.97157°N	114.38408°W
Elko	BC, CAN	49.29157°N	115.12134°W
Gyor	Hungary	47.67809°N	17.63929°W

20  $\mu$ L of 4 mg/mL DMACA in ethanol. Values obtained using the DMACA method were then compared to those obtained using the standard HPLC protocol described previously (Perry et al., 2007).

### 2.3. Soil extraction

Soil was collected from beneath grasses growing near a *C. maculosa* patch on Mt. Sentinel (Table 1). The soil did not support *C. maculosa*, but was of a similar soil type to that beneath the adjacent *C. maculosa* patch. The soil was air-dried and homogenized before use. 40 g of soil (~50 mL volume) was spiked with 0, 5, or 15.0 mg of (+/-)-catechin (Sigma, C1788) for use in extractions. Dry catechin was suspended in 2 mL distilled water and immersed in a sonicating water bath to break catechin agglomerates into fine particles. The aqueous catechin suspension (catechin is not soluble in water at these concentrations) was added to dry soil. The vials were then rinsed in an additional 2 mL distilled water and this was added to the soil as well. This resulted in 40 g of soil with 4 mL of water, allowing realistic soil moisture conditions to be tested. Catechin-free control soil was also moistened with 4 mL water. The catechin-spiked soil was mixed thoroughly by hand for 10 min to achieve as homogenous a distribution as possible.

Moistened soil (0.3 g) was transferred to a 2.0 mL eppendorf tube. The spiked soil was then extracted with 1.5 mL of either ethanol, methanol, ethanol+1% HCl, or methanol+1% HCl. The samples were extracted by vortexing for 15 s. After extraction, the samples were centrifuged for 10 min at 13,000 rpm with a benchtop centrifuge. 180  $\mu$ L of the particle-free supernatant was then transferred to a 96-well plate. 20  $\mu$ L of 4 mg/mL DMACA was then added to each well and 2  $\mu$ L of concentrated HCl was added to the ethanol and methanol extraction samples (to achieve the same final HCl concentration). The solution was allowed to develop for 20 min at room temperature before measuring absorbance. The absorbance values were compared to those obtained for authentic (+/-)-catechin from 0.0 to 100  $\mu$ g/ $\mu$ L final catechin concentration. 100% recovery would yield a final concentration of 23.8  $\mu$ g/mL catechin in the DMACA assay solution for the 15 mg dose, and 7.9  $\mu$ g/mL for the 5 mg dose. Percent recovery was calculated as the actual amount recovered divided by the theoretical 100% recovery amount times 100.

To test for the effect of water content of the soil, 10 mg of catechin was suspended in either 0.5, 1.0 or 1.5 mL of water. After vortexing and sonicating to create a uniform suspension, the water with catechin was applied to 10 g of soil. The vials were then rinsed with a second volume for a total water content of 1.0 mL/10 g soil (low), 2.0 mL/10 g soil (moderate), or 3.0 mL/10 g soil (high water content). These soils were mixed thoroughly by hand to achieve a uniform catechin distribution. The weight of moistened soil was adjusted such that each extracted sample contained the same amount of soil and catechin—low samples contained 0.33 g soil+water, moderate contained 0.36 g soil+water,

and high contained 0.39 g soil+water. Thus the only change was in the water content, and the total soil and catechin was the same for each sample. The samples were extracted with 1.5 mL of 1% HCl in ethanol by vortexing for 10 s and centrifuging for 5 min. 180  $\mu$ L of this solution was added to 20  $\mu$ L of 4 mg/mL DMACA in ethanol, allowed to develop for 20 min, and the absorbance measured at 600 nm. The remaining extract (0.75 mL) was reserved for HPLC–MS analysis. LC–MS analysis was conducted on a Dionex system composed of P680 pump, and ASI-100 autosampler, and a PDA100 photodiode array detector. This was coupled to a Thermo Finnigan Surveyor MSQ mass spectral detector. Separation was performed on a Dionex Acclaim 120 C18 column (5  $\mu$ m, 4.6  $\times$  150 mm<sup>2</sup>) using gradient elution. Solvent A was water+0.1% v/v acetic acid and solvent B was methanol+0.1% acetic acid. Compounds were eluted at a 0.7 mL/min flow rate for 3 min at 10% B, a linear gradient to 90% B over 40 min, and held at 90% B for 8 min. UV detection was recorded from 200 to 800 nm. Ionization for MS analysis was performed in both positive and negative ion mode using electrospray ionization with a nitrogen flow at 80 psi, a cone voltage of 70 V, needle voltage of 3 kV, and sheath temperature of 600 °C. Mass data were collected over the range of the gradient program at a rate of one scan per 2 s.

### 2.4. Statistical analysis

Raw absorbance values were log transformed for both graphical display and analyses of variance (ANOVA). ANOVA and Tukey's HSD post-hoc comparisons were performed in JMP v 5.1.2 (SAS Institute, Cary, North Carolina, USA).

## 3. Results

### 3.1. Assay development

An alcohol solution of DMACA is yellow in color, and changes to blue in color in the presence of catechins. To guide the assay protocol development, the UV–visible absorbance spectrum was acquired for the reagent solution in the presence and absence of catechin. This experiment revealed a large absorbance peak in the presence of catechin at 640 nm, which was not present in the absence of catechin (Fig. 1). Though monitoring at 640 nm would provide optimal sensitivity, we utilized a plate reader equipped with a 600 nm filter—absorbance is approximately 50% maximal at this wavelength. A laboratory capable of monitoring at 640 nm could increase the sensitivity of this assay further. All subsequent assays are quantified using 600 nm absorbance. The DMACA solution in the absence of catechin has absorbance values at 600 nm that are indistinguishable from solvent controls that did not contain DMACA.

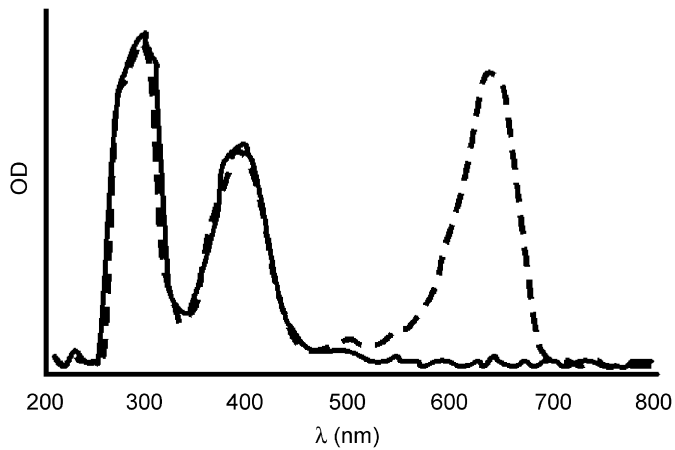


Fig. 1. UV-visible light absorption spectrum for DMACA catechin assay solution (dashed line) of 8 parts 5% HCl in ethanol (v/v), 1 part 2.0 mg/mL DMACA in ethanol, and 1 part 100  $\mu\text{g/mL}$  (+/-)-catechin hydrate in ethanol. The solid line represents the same solution, except pure ethanol replaced catechin solution.

Several DMACA concentrations were examined to determine maximal sensitivity, taking into consideration sensitivity as measured both by spectrophotometric readings at 600 nm and visible examination. All DMACA concentrations tested demonstrated response linearity between 0.01 and 10  $\mu\text{g/mL}$  final concentration, a dynamic range of four orders of magnitude (Fig. 2a). Visible sensitivity was slightly less, with 0.1  $\mu\text{g/mL}$  of catechin being the minimum concentration reliably differentiated from the catechin-free control. Catechin concentrations of 0.01  $\mu\text{g/mL}$  could often be distinguished from controls, but with less confidence. Visible sensitivity was greater when higher concentrations of DMACA were used. For subsequent assays, 20  $\mu\text{L}$  of a 4.0 mg/mL DMACA stock solution was used in a reaction solution of 200  $\mu\text{L}$ .

HCl is highly toxic, and minimization of HCl levels will allow for a safer field assay. HCl concentrations were systematically varied to minimize its concentration while retaining sensitivity. All HCl tested concentrations demonstrated strong response linearity and at all HCl concentrations the lowest catechin concentration tested (0.01  $\mu\text{g/mL}$ )

was clearly distinguishable from catechin-free controls (Fig. 2b). However, lower HCl concentrations (1% HCl v/v) gave slightly but consistently higher absorbance readings than did higher (5% and 10% HCl v/v) concentrations. HCl (1%) was used for further assays.

The DMACA colorimetric reaction begins to develop immediately, but is not complete for some time. To provide data to infer the minimal and optimal incubation time, an experiment was performed that monitored the development of absorbance at 600 nm over time at the four intermediate

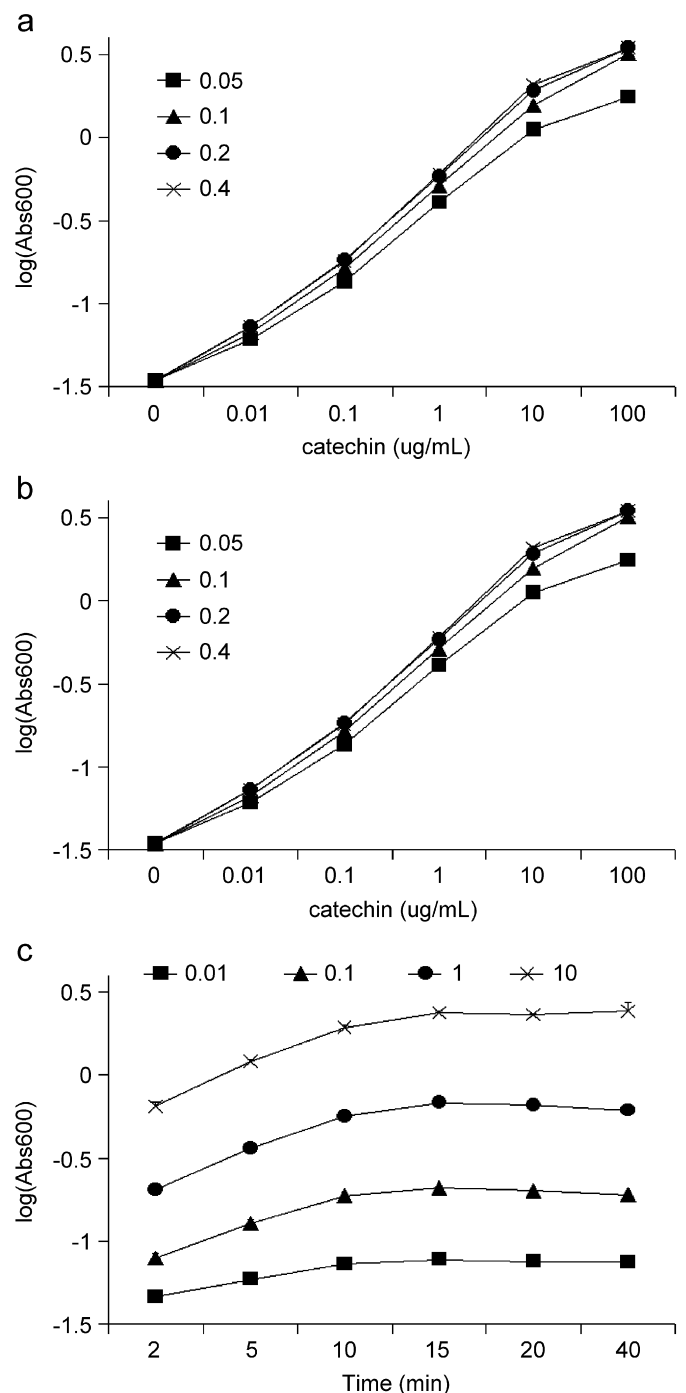


Fig. 2. Assay development for maximal sensitivity: (a) All DMACA concentrations tested were sensitive enough to distinguish 0.01  $\mu\text{g/mL}$  of (+/-)-catechin from the catechin-free control, and all concentrations were distinguishable from each other. DMACA concentrations of 0.2 and 0.4 mg/mL (final concentration) are more sensitive than 0.1 and 0.05 mg/mL at all catechin concentrations except the catechin-free control. Visible sensitivity was greatest at higher DMACA concentrations (0.4 and 0.2 mg/mL), at which even the lowest catechin concentrations were often visually distinguished from catechin-free controls. (b) All catechin concentrations were distinguishable from catechin-free controls at all HCl concentrations. HCl (1%) was more sensitive than higher concentrations at concentrations from 0.01 to 10  $\mu\text{g/mL}$ . (c) Development is essentially complete at 15 min, and absorbance begins to fall slightly after 40 min. Different symbols indicate different (+/-)-catechin concentrations as described by the legends. Error bars represent standard error, but are often concealed beneath the symbols.  $n = 4$  for all data points.

catechin concentrations (0.01–10.0 µg/mL) in a solution of 1% HCl and 4% DMACA. The reaction was performed at room temperature (22 °C) and monitored for 40 min. The data demonstrated that the absorbance is maximal at approximately 15–20 min, and thereafter begins to decay very slightly (Fig. 2c). Over longer time frames (> 12 h), the blue pigmentation fades and a red coloration begins to develop (not shown). Thus for quantitative purposes, optimal sensitivity is achieved between 15 and 20 min, though this time would likely decrease with increasing temperature. The subtle decay after 20 min is not detectable visibly, thus visual examination for qualitative purposes can be reliably scored between 15 min and 2 h.

### 3.2. Assay validation and extraction efficiency

To test whether the laboratory results would translate to a successful field assay, samples that had previously been prepared from field collected samples from *C. maculosa* patches from multiple soils were tested (Perry et al., 2007). These included samples previously demonstrated to contain catechin and those that did not. This allowed for a test of the sensitivity of the assay, to compare the quantitative results with those previously obtained using a more established HPLC-based method, and to determine whether other soil components may cross-react with the reagent to generate false-positive results. All samples that were found to contain no catechin as previously determined by HPLC analysis demonstrated absorbance values at 600 nm below those for the lowest standard catechin concentration used (<0.01 µg/mL—data not shown). These samples were collected from numerous sites with diverse soil properties (Table 1), demonstrating that false-positive results are likely to be rare.

All samples that were found to contain catechin by HPLC analysis were also found to contain catechin by DMACA detection. The quantitative results as calculated by detection with DMACA and compared to a catechin dose–response curve were in general agreement with the HPLC results, but the exact quantities did deviate from the HPLC results, in one instance rather dramatically (Fig. 3). These differences are likely due to components of the soil extract, which interfere with the colorimetric reaction. It is currently unclear what those components might be.

Catechin extraction efficiency was then tested from field collected soils. 40 g of air-dried soil was spiked with either 0, 5, or 15 mg of (+/–)-catechin, moistened with 4 mL of water, mixed thoroughly, and subsamples extracted with one of four alcohol-based solvent systems: ethanol, ethanol+1% HCl, methanol, or methanol+1% HCl. Catechin-free samples were used to estimate the background absorbance generated by extraction with each solvent system. Inclusion of HCl in the methanolic or ethanolic extraction increased the color of the extract, both visually and as measured by absorbance at 600 nm (Fig. 4a). This generated a brown solution, which did not appear blue visually, thus is not likely to be confused as a

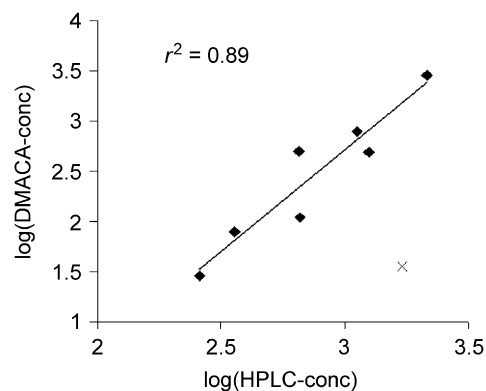


Fig. 3. The DMACA method was applied to previously collected field soil methanol extracts. Plotting the quantities obtained with each method demonstrates strong agreement, with the exception of the sample represented by the 'X' symbol. If this sample is included, the  $r^2$  values drops to 0.36. Note also that the slope of the line is not 1; at lower concentrations, the DMACA method underestimates catechin concentration.

positive catechin test. Ethanol+1% HCl was more effective for extracting catechin than was ethanol alone, but less effective than methanol (Fig. 4b). Methanol is slightly more effective than ethanol at higher concentrations (15 mg catechin per 40 g soil), but less effective at lower concentrations (5 mg/40 g soil), and methanol+HCl is more effective than is ethanol+HCl at lower concentrations, but not at higher concentrations. In all cases, extraction efficiency was low, with maximal values of ~5%.

Field soil moisture conditions vary considerably seasonally, from day to day and within a day. It is therefore informative to compare the extraction efficiency and reagent sensitivity under different soil water conditions. Soil spiked with catechin was wetted to low, moderate, or high water content (1, 2, or 3 mL water per 10 g soil) and a sample of the soil was extracted with 1.5 mL 1% HCl in ethanol. 180 µL of this extract was transferred to a 96-well plate and mixed with 20 µL 4 mg/mL DMACA in ethanol. The absorbance at 600 nm demonstrated less color development with higher soil water content. HPLC analysis of the same extracts (not shown) demonstrated that the reduced absorbance at 600 nm was due not to reduced extraction efficiency, but to reduced color development in the presence of higher water content, as previously demonstrated (McMurrough and McDowell, 1978; Treutter, 1989).

## 4. Discussion

This novel method offers a rapid, highly sensitive qualitative assay for analysis of soil catechin under field conditions. The assay is somewhat sensitive to soil water content and interference from unknown soil components, thus quantitative results based solely on absorbance at 600 nm should be viewed with some caution. At this time,

we must conclude that use of an HPLC should be considered the superior method for quantitative results, as separation helps to reduce interference.

Catechin extractions from soil are typically performed with methanol or acetone. Acetone has been previously

shown to inhibit color development (Treutter, 1989). Methanol is compatible with DMACA reaction conditions, but is relatively toxic—a poor trait for an assay with potential use under field conditions. Ethanol was found to offer similar extraction efficiency and serves as a relatively non-toxic assay solvent. Likewise, HCl is toxic at high concentrations, and its concentration was adjusted to reduce toxicity while retaining assay sensitivity. The results from these tests suggest that ethanol+1% HCl offers the best option, balancing extraction efficiency at both high and low concentrations, DMACA color development for maximal sensitivity, and safety and environmental concerns. DMACA itself is relatively non-toxic, and maximal sensitivity was found at higher concentrations (a final assay concentration of 0.4 mg/mL working from a 4.0 mg/mL stock solution).

Extraction efficiency is low under all conditions tested. This has been previously observed for alcohol-based extraction methods in Montana soils (Blair et al., 2005), and the results obtained for the DMACA study are in general agreement with the previous reports. DMACA is reliant on alcohol as a reaction solvent and the reaction is sensitive to water, thus the acetone/water solvent system (approximately 33% extraction efficiency in soils similar to those used for this study) previously described (Blair et al., 2005) is infeasible. The poor extraction efficiency of catechin from soils could be due to chelation by di- or tri-valent cations and/or pH sensitive degradation. Previous reports describe significantly higher recovery from dry soil and from sand (Blair et al., 2005), suggesting that catechin in aqueous solution either degrades or becomes resistant to alcohol-based extraction due to interaction with soil components. These recovery issues are potentially limiting when absolute quantitation is necessary and a relatively insensitive assay is used. However, the sensitivity of the DMACA assay allows for reliable detection despite the poor extraction efficiency of alcohol-based solvents. For example, catechin concentrations of 50  $\mu\text{g/g}$  soil with an extraction efficiency of 1% would generate a solution concentration of 0.1  $\mu\text{g/mL}$ —a concentration that is readily detected through either use of a spectrophotometer or visual examination. In fact levels 10 times lower than this are still readily detected by a spectrophotometer and can often be distinguished visually. For comparison, recent studies indicate that when catechin is present, it is present at levels between 140 and 2150  $\mu\text{g/g}$  soil (Perry et al., 2007),

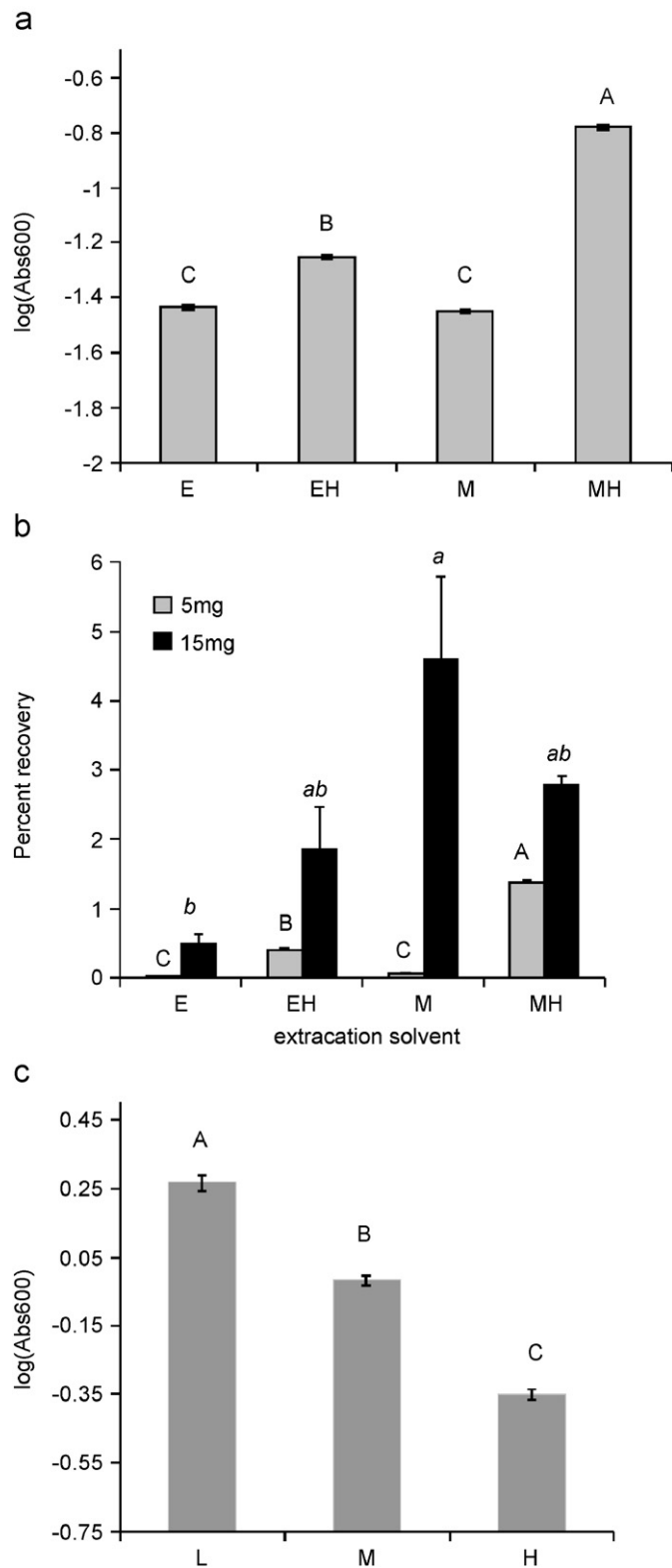


Fig. 4. Comparison of solvents on extraction and detection. (a) Comparison of absorbance at 600 nm when extracting from catechin-free soil with ethanol (E), ethanol+1% HCl (EH), methanol (M), or methanol+1% HCl (MH). Error bars represent standard error. Bars labeled with the same letter are not significantly different (Tukey's HSD,  $p < 0.05$ ). (b) Effect of various solvents on extraction efficiency—labeled as in part (a). (c) Increased soil water content results in reduced DMACA development in a manner that is dose dependent. Low (L—1 mL/10 g soil), moderate (M—2 mL/10 g soil) and high water content (H—3 mL/10 g soil).

indicating that levels of catechin found under field conditions can be detected using the DMACA method, despite low extraction efficiency.

For field assays, we recommend use of a freshly prepared solution of 1% HCl in ethanol and 4.0 mg/mL DMACA in ethanol. A small amount (<0.5 g) of soil should be added to a 2.0 mL eppendorf tube containing 1.5 mL of acidic ethanol extraction solution. The soil extract should be vigorously shaken, and allowed to extract for ~10 min while shielded from light; during this time soil particles will fall out of solution if left undisturbed. 450  $\mu$ L of this solution can be transferred to a new tube, and 50  $\mu$ L of the DMACA solution added to the soil extract (volumes can be adjusted as necessary, so long as the proportions remain the same). High catechin concentrations will generate a visible blue color almost instantly, and maximal sensitivity will typically be achieved within 20 min. The remaining soil extract can be transported back to the lab if the DMACA assay demonstrates a positive test and the remaining soil, or an adjacent soil sample, extracted more thoroughly using previously established methods if precise and absolute quantitation is desired. Alternatively, the entire reaction solution (1% HCl and 0.4 mg/mL DMACA in ethanol) could be used as an extraction solvent. Previous reports indicate that a solution of DMACA in acidic alcohol is stable for up to a week if kept in the dark (McMurrough and McDowell, 1978; Treutter, 1989); thus containers in which this solution is stored should be kept from light. In this instance, there is no need to transfer from tube to tube; the solution will develop as the catechin extraction proceeds. In this case, a second soil sample should be collected for subsequent HPLC analysis, as the stability of catechin as a DMACA conjugate is unknown.

This method will allow for a rapid screen to guide the sampling process and allow for increased sampling that may elucidate the factors that affect soil catechin levels. The unpredictability of the presence and quantity of catechin may be due to a lack of knowledge of the regulation of its temporal production or secretion or factors influencing its degradation in soils. This efficient and rapid sampling method will allow for greater sampling regularity and more diverse sampling sites. The data generated using this method may help to clarify the regulation of catechin production by *C. maculosa* and potentially clarify the role of this putative allelochemical in *C. maculosa* invasion.

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