

## *Colletotrichum coccodes* and thidiazuron alter specific peroxidase activities in velvetleaf (*Abutilon theophrasti*)

R. G. NICKERSON\*, T. J. TWORKOSKI† and D. G. LUSTER‡

U.S.D.A. Agricultural Research Service, \* Foreign Disease-Weed Science Research Unit, Frederick, MD 21702; † Appalachian Fruit Research Station, Kearneysville, WV, 25403, U.S.A.

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The height and biomass of velvetleaf (*Abutilon theophrasti*), were inhibited by treatment of young seedlings with the fungal pathogen *Colletotrichum coccodes* or with the growth regulator thidiazuron (TDZ). Applied simultaneously, these two agents synergistically reduced velvetleaf height and biomass. The use of two different peroxidase substrates, guaiacol and *o*-dianisidine, detected different peroxidase isoform activities. This study identified *C. coccodes*- and TDZ-induced changes in peroxidase activity and isoform pattern.

Treatment with *C. coccodes* increased soluble peroxidase activity as measured spectrophotometrically with the peroxidase substrate guaiacol. TDZ treatment had no observed effect on guaiacol-reactive soluble peroxidase activity. Isoelectric focusing analysis of peroxidase isoform activity using *o*-dianisidine as a substrate demonstrated that both TDZ and *C. coccodes* caused changes in the temporal expression and activity of specific anionic isoforms. TDZ enhanced the activity of a pI 4.26 isoform while *C. coccodes* enhanced the activity of a pI 4.03 isoform. The combination of TDZ and *C. coccodes* caused a simultaneous and rapid stimulation of both these isoforms. Based on these observations, and previously published reports of disease symptomatology, we propose that the increase in guaiacol-reactive soluble peroxidase activity represents a peroxidase-associated defence response, and that the increased activity of the pI 4.03 and 4.26 isoforms is associated with the inhibition of growth. The rapid stimulation of both isoforms following the combined treatment may account for the synergistic inhibition of growth.

### INTRODUCTION

The combined application of plant growth regulators with biological control agents is a potentially powerful strategy for the integrated management of weeds. The goal of this approach is to disrupt the growth habits and defence mechanisms of the target weed so that it becomes more susceptible to the biological control agent.

The malvaceous weed velvetleaf (*Abutilon theophrasti* Medik.) is an aggressive competitor in soybean and corn fields and can significantly reduce the yields of these crops [22]. The fungal pathogen *Colletotrichum coccodes* has been identified as a potential biological control agent against velvetleaf [20]. *C. coccodes* frequently causes infected leaves to abscise, resulting in the inhibition of weed growth and sometimes weed mortality [27]. The growth regulator thidiazuron (TDZ) is a cytokinin analogue [28]

‡To whom correspondence should be addressed.

Abbreviations: IEF, isoelectric focusing; pI, isoelectric point; TDZ, thidiazuron; ai, active ingredient.

that inhibits velvetleaf growth when applied in low doses [12]. Initial field experiments confirmed that combined application of *C. coccodes* spores and TDZ, applied to very young velvetleaf seedlings, synergistically reduced both shoot height and biomass, and increased mortality [11, 26]. Field applications of *C. coccodes* and TDZ had no adverse effect on soybean or corn crop yields [11].

The mechanisms by which *C. coccodes* and TDZ suppress velvetleaf growth, acting singly or synergistically, are unknown. However, combined treatments of *C. coccodes* and TDZ synergistically enhanced ethylene production in velvetleaf seedlings [10]. Increased endogenous ethylene production or exogenously applied ethylene, has been associated with an increase of peroxidase enzymes in cucumber [1, 3], sweet potato [23], tobacco [16], barley [9] and tomato [21].

Peroxidases (EC 1.11.1.7) are ubiquitous plant enzymes implicated in the control of plant growth and in plant response to pathogen challenge [8, 17]. Recent reports have documented an inverse relationship between peroxidase activity and shoot elongation in tomato [13] and peanut [28]. Tobacco plants transformed with cDNA coding for two anionic peroxidase isoforms displayed a wilting phenotype and stunted growth [15].

An increase in peroxidase activity and/or an alteration of expressed isozymes is characteristic of plant defence in response to pathogen challenge [8, 17]. Resistant cultivars typically have higher constitutive peroxidase levels, or demonstrate a more rapid increase in peroxidase activity in response to pathogen challenge compared to their near-isogenic susceptible relatives [14, 19, 24].

We therefore postulated that inhibition of growth in response to treatment with TDZ and *C. coccodes* might be associated with changes in peroxidase activity. In this study, alterations in peroxidase activity and isoform expression in velvetleaf plants responding to *C. coccodes* infection and/or the growth regulator TDZ were characterized in an attempt to understand the biochemical mechanisms underlying their synergistic interaction.

## MATERIALS AND METHODS

### *Biological material*

Wild-type velvetleaf seeds were germinated on Whatman 1 filter paper saturated with sterile deionized water in sealed Petri dishes. The sealed Petri dishes were incubated at 4 °C for 48 h and then transferred to the growth chamber and incubated an additional 48 h until the seed coats cracked. Germinated seeds were planted in 4 inch pots at a density of four per pot and thinned to three per pot when the cotyledons were fully expanded. Seedlings were maintained in the growth chamber with a 14 h photoperiod at 24 °C and 54% RH. Three hundred  $\mu\text{E}\cdot\text{m}^{-2}$  irradiance was provided by a combination of cool white fluorescent and incandescent bulbs during the light period. Dark period conditions were set to 18 °C and 66% RH.

*Colletotrichum coccodes* (AGII isolate) was maintained on solid V-8 medium [4] at 25–26 °C. For spore production 3 mm agar plugs were inoculated into 250 ml V-8 liquid medium and grown at 24–26 °C on a rotary shaker. Spores were harvested 7 days after inoculation [26].

*Spray treatments*

Harvested fungal spores were resuspended in sterile deionized water and counted with a hemacytometer. Thidiazuron (*N*-phenyl-*N'*-1,2,3,-thiazol-5-yl-urea) was commercially supplied as DROPP (Nor-am Chemical Company, Wilmington, DE, U.S.A.). DROPP and *C. coccodes* spores were suspended in sterile deionized water and applied to 1–2 leaf stage velvetleaf seedlings in a spray chamber (Richard Scientific Inc., Novato, CA, U.S.A.) equipped with a moving spray nozzle. To ensure consistency, *C. coccodes* spores and DROPP was applied on the basis of number of spores or mass respectively per unit surface area of the spray chamber ( $\text{mg ai m}^{-2}$ ). Control plants were sprayed with sterile deionized water. All plants were immediately transferred to a dew chamber set to 21 °C and 100% RH and incubated in the dark for 20 h. Plants were then returned to the growth chamber for the duration of the experiment.

*Measurement of velvetleaf shoot height and biomass*

Velvetleaf seedlings were maintained in the growth chambers for 21 days after spray treatment. On day 21 post-treatment, shoot height was measured from the cotyledonary scar to the uppermost leaf node. Shoots were excised at the cotyledonary scar and dried at 70 °C for 72 h before weighing. Nine plants (three pots) were used to generate the average height and biomass for each treatment. An analysis of variance was conducted with mean separation based on Fisher's protected LSD.

*Extraction of soluble peroxidases and intercellular fluid*

The first and second leaves immediately above the cotyledons (leaves 1 & 2) were excised from each plant in a pot and the six leaves from a single pot were combined for extraction. Freshly harvested leaves were weighed and then frozen in liquid  $\text{N}_2$  and ground to a dry powder with mortar and pestle. The powder was transferred to a conical polypropylene tube and 7 ml of 50 mM sodium acetate (pH 5.0) were added for each gram (fresh weight) of plant tissue. The leaf suspension was incubated on ice and vortexed vigorously at 10 min intervals for 30 min. The suspension was filtered through miracloth and the filtrate was retained. The brei was re-extracted with an additional volume of sodium acetate (pH 5.0). Filtrates were combined and centrifuged at 10000 *g* for 10 min using an SS-34 rotor in a Sorvall RC5B centrifuge. The resulting supernatant was concentrated and washed with 50 mM sodium acetate buffer (pH 5.0) using Amicon (Beverly, MA, U.S.A.) Centriprep-30 or Centricon-30 concentrators. This procedure removes low molecular weight substrates and inhibitors below 30 kDa. The concentrates were reconstituted to 10 ml with 50 mM sodium acetate (pH 5.0) before use in the peroxidase assay.

Intercellular wash fluid was obtained according to Kerby and Somerville [14] except that the cut leaves were rolled in plastic film and inserted into an empty 6 cc syringe for support. The entire syringe was placed in a conical centrifuge tube and centrifuged in a Beckman GP centrifuge equipped with a swinging bucket rotor at 1000 *g* for 30 min at 4 °C. All extracts were stored at –20 °C.

*Peroxidase assay*

Peroxidase activity was measured in a dual beam spectrophotometer at 470 nm in a 1.0 ml assay mixture containing 40 mg total protein, 0.3% guaiacol, 0.3%  $\text{H}_2\text{O}_2$  and

50 mM sodium acetate (pH 5.0). Each extract was assayed three times to achieve an average value for the sample. The data presented represent an average of five experimental trials. Regression analysis was used to determine the peroxidase activity response over time. Analysis of variance and Fisher's protected LSD were conducted so that peroxidase activities for each treatment and time could be compared. Protein content of plant extracts was determined by the Markwell [18] procedure using gammaglobulin (Bio-Rad, Richmond, CA) as a standard. All extracts were tested in a blank reaction mixture containing no exogenously added substrate or peroxide. No peroxidase activity was detected under these conditions.

#### *Isoelectric focusing polyacrylamide gel electrophoresis*

Leaves 1 and 2 were harvested from all plants in five pots and extracted for peroxidase activity as above. The data presented were generated in a single 13 day experiment. Extracts were successively vacuum filtered through Whatmann GF/M fibreglass and 0.45  $\mu\text{m}$  nylon filters. Filtrates were concentrated and washed with 50 mM sodium acetate (pH 5.0) using either Amicon Centriprep-30 or Centricon-30 concentrators. The peroxidase activity in the concentrates was determined per microlitre, using guaiacol as a substrate. Samples containing 1.0 OD  $\text{min}^{-1}$  peroxidase activity were run on the pHast system (Pharmacia, Piscataway N.J., U.S.A.) using prepoured IEF media pH 3-9 according to the manufacturer's specifications. Gels were incubated at room temperature for 15 min in 20 ml of reagent containing 0.3%  $\text{H}_2\text{O}_2$ , 50 mM sodium acetate (pH 5.0), and 0.2 mg  $\text{ml}^{-1}$  *o*-dianisidine or 0.3% guaiacol as a substrate. This reagent was then poured off and the gels were washed and stored in deionized distilled water. Standards supplied by Pharmacia for use with the pHast system were used to calculate the isoelectric point (pI) of isoforms.

## RESULTS

### *Effects of TDZ and C. coccodes on velvetleaf shoot height and biomass*

All concentrations of TDZ applied without *C. coccodes* reduced the shoot height of velvetleaf (Table 1). TDZ applied without *C. coccodes* reduced biomass only at the highest concentration applied in this experiment (0.64 mg  $\text{ai m}^{-2}$ ) (Table 1). *C. coccodes* without TDZ was only effective in reducing shoot height or biomass of velvetleaf at the highest inoculum levels applied ( $24 \times 10^7$  spores  $\text{m}^{-2}$ ) (Table 1). All combinations of TDZ and *C. coccodes* reduced the height and biomass more than the sum of each treatment alone.

The combination of TDZ at 0.32 mg  $\text{ai m}^{-2}$  and *C. coccodes* at  $2.4 \times 10^7$  spores  $\text{m}^{-2}$  was the minimum dose determined to be sufficient for consistent synergistic reduction of both shoot height and biomass. This combination was used in all subsequent experiments for the determination of peroxidase response to treatment with TDZ and *C. coccodes*. Seedling mortality was not observed in any treatments tested during these experiments (data not shown). The concentrations of TDZ and *C. coccodes* used in this growth chamber study were an order of magnitude less than those found to have similar effects on the shoot height and weight of velvetleaf in field studies [26].

TABLE 1

*Effect of thidiazuron and C. coccodes on shoot height and biomass in velvetleaf. Significant interaction between TDZ and C. coccodes prohibited simplified averaging of main effects. Mean separation was conducted based on Fisher's protected least significant difference*

TDZ (mg ai m <sup>-2</sup> )	<i>C. coccodes</i> (spores m <sup>-2</sup> × 10 <sup>7</sup> )	Average height (cm)	Average weight (g)
0	0	22.0	1.00
0	0.24	21.3	1.06
0	2.4	23.6	1.28
0	24.0	19.8	0.89
0.16	0	19.3	0.98
0.16	0.24	13.2	0.38
0.16	2.4	8.7	0.21
0.16	24.0	12.8	0.29
0.32	0	14.6	0.92
0.32	0.24	8.9	0.20
0.32	2.4	8.7	0.21
0.32	24.0	8.4	0.19
0.64	0	12.3	0.64
0.64	0.24	11.5	0.33
0.64	2.4	7.9	0.19
0.64	24.0	9.2	0.27
LSD = 0.05		1.93	0.10

*Effects of C. coccodes and TDZ on soluble peroxidase activity in velvetleaf seedlings*

Soluble guaiacol-reactive peroxidase activity increased with time in all seedlings regardless of treatment (Fig. 1). Spray treatment of 1–2 leaf stage seedlings with TDZ alone did not significantly affect soluble peroxidase activity. Application of *C. coccodes* significantly increased soluble peroxidase activity on day 13 post-treatment (experimentwise LSD 0.05 = 4.35). No significant difference in peroxidase activity between TDZ and TDZ + *C. coccodes*-treated plants was found in this study.

*Alteration of soluble peroxidase isoform expression in C. coccodes- and TDZ-treated velvetleaf seedlings*

Isoelectric focusing of soluble extracts from plants treated with both TDZ and *C. coccodes* followed by incubation of gels with *o*-dianisidine as a substrate for peroxidase activity produced 15 isoforms ranging in pI from 4.03 to 8.66 (Fig. 2, lane a). In contrast to the pattern observed with *o*-dianisidine, the use of guaiacol as a substrate detected four isoforms (pI 4.26, 4.82, 5.19, and 8.66) (Fig. 2, lane b). Three isoforms (pI 4.82, 5.19, and 8.66) were detected in the intercellular wash fluid using either *o*-dianisidine (Fig. 2, lane c) or guaiacol (data not shown) as substrates. The presence of additional *o*-dianisidine reactive isoforms in the soluble extract which did not use guaiacol as a substrate indicated that the spectrophotometric data gathered using guaiacol (Fig. 1) underestimated the total soluble peroxidase activity. The majority of isoforms detected by guaiacol in the soluble extract appear to be intercellular. Therefore, guaiacol as a substrate predominantly measured changes in intercellular peroxidase activity.

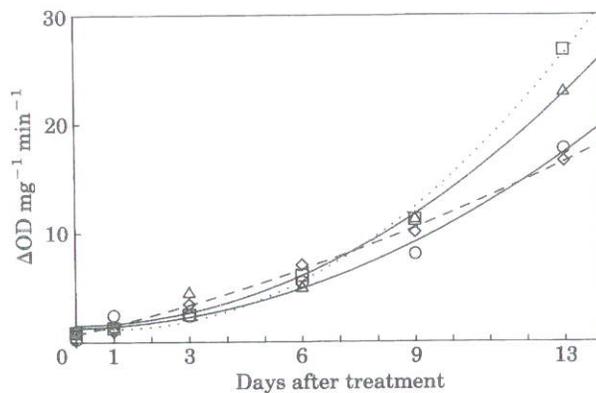


FIG. 1. Effect of TDZ ( $0.32 \text{ mg ai m}^{-2}$ ) and *C. coccodes* ( $2.4 \times 10^7$  spores  $\text{m}^{-2}$ ) treatment of velvetleaf on guaiacol-reactive soluble peroxidase activity. Control (—○—),  $y = 1.28 + 0.07x + 0.09x^2$ ,  $R^2 = 0.99$ ; TDZ (---◇---),  $y = 0.69 + 0.81x + 0.03x^2$ ,  $R^2 = 0.99$ ; *C. coccodes* (...□...),  $y = 1.37 - 0.34x + 0.17x^2$ ,  $R^2 = 0.99$ ; TDZ + *C. coccodes* (·-·△-·-·),  $y = 1.54 + 0.02x + 0.12x^2$ ,  $R^2 = 0.99$ .

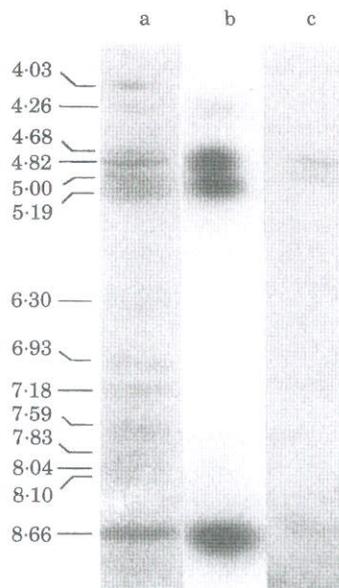


FIG. 2. Soluble peroxidases in TDZ ( $0.32 \text{ mg ai m}^{-2}$ ) + *C. coccodes* ( $2.4 \times 10^7$  spores  $\text{m}^{-2}$ )-treated velvetleaf seedlings extracted 13 days following treatment and separated on IEF media pH 3–9. (a) incubated with *o*-dianisidine as a substrate; (b) incubated with guaiacol as a substrate; (c) intercellular fluid incubated with *o*-dianisidine as a substrate. pH values of specific bands are listed on the left.

Changes in the peroxidase isoform pattern caused by TDZ and/or *C. coccodes* treatment became apparent when *o*-dianisidine was used as a substrate. Both TDZ and *C. coccodes* treatments stimulated distinct anionic *o*-dianisidine-reactive peroxidase isoforms (Fig. 3)

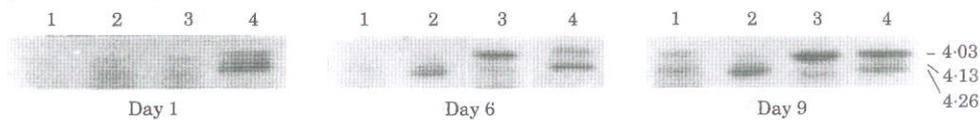


FIG. 3. Soluble peroxidase isoforms as separated on IEF media pH 3–9 in control (lane 1), TDZ-treated (lane 2), *C. coccodes*-treated (lane 3), TDZ + *C. coccodes*-treated (lane 4) velvetleaf extracts on day 1, day 6, day 9 following treatment. pH values of specific bands are listed on the right.

Treatment of velvetleaf with TDZ, alone or combined with *C. coccodes*, stimulated the activity of the pI 4.26 isoform (Fig. 3). TDZ also induced the appearance of a novel isoform at 4.13 in conjunction with the pI 4.26 isoform (Fig. 3) although this isoform was only weakly visible. The enhancement of pI 4.26 and 4.13 isoforms, in response to TDZ treatment without *C. coccodes*, although sometimes detectable on the first day following treatment, did not become consistently apparent until the sixth day following treatment.

Treatment of plants with *C. coccodes*, either alone or in combination with TDZ, stimulated the activity of the pI 4.03 isoform (Fig. 3). The enhancement of pI 4.03 activity, in response to *C. coccodes* treatment without TDZ, was sometimes detectable on the first day following treatment, although it did not become clearly apparent until the sixth day following treatment (Fig. 3). *C. coccodes* did not stimulate the pI 4.26 isoform, nor did it cause the induction of the pI 4.13 isoform. The pI 4.03 isoform stimulated by *C. coccodes* treatment was not stimulated by treatment with TDZ.

The combined application of *C. coccodes* and TDZ resulted in alterations of temporal expression and identity of isoforms observed compared to plants treated with either TDZ or *C. coccodes* alone. On the first day following treatment, the combination of TDZ and *C. coccodes* resulted in the strong and rapid enhancement of all three anionic isoforms (Fig. 3). In contrast, these anionic isoforms were not enhanced by either treatment alone until the sixth day following treatment. IEF analysis of mycelial extracts of *C. coccodes* did not produce any isoforms at pI 4.03, 4.13 or 4.26 (data not shown).

The activity of these anionic peroxidases increased as the plant aged and growth began to slow, or following treatment with TDZ and/or *C. coccodes*, agents which inhibited growth (Fig. 3). The IEF data presented concur with the observations in previous reports [13, 15, 29] that anionic peroxidase activity inversely correlates with growth.

## DISCUSSION

The use of two different peroxidase substrates in these studies provided information about specific isoperoxidases and their association with both pathogen, and growth regulator-induced responses. Soluble peroxidase activity was quantitated spectrophotometrically using guaiacol as a substrate. Plants treated with the fungal foliar pathogen *C. coccodes* display increased guaiacol reactive peroxidase activity. IEF analysis using both guaiacol and *o*-dianisidine as substrates indicates that guaiacol reacts with predominantly intercellular peroxidase isoforms. Based on these data and

other reports of pathogen-induced increases in peroxidase activity [8, 14] we propose that this spectrophotometric analysis indicates a peroxidase-associated defence response of velvetleaf.

In contrast to the rapid pathogen-induced plant defence responses reported in barley [14], and cowpea [6], the velvetleaf (1–2 leaf stage) response to pathogen challenge is sustained over a period of days rather than hours. The time course of peroxidase response is approximately coincident with the development of *C. coccodes* induced disease symptoms. Although stimulation of the pI 4.03 isoform by *C. coccodes* is barely detectable 24 h following treatment, it is clearly evident by the sixth day following treatment (Fig. 3). Lesions first appear on the leaves of *C. coccodes* infected velvetleaf 4–5 days following inoculation. Infected leaves may begin to abscise soon thereafter. If the plant is not killed as a result of the infection, it will continue to grow although its growth may be stunted [27].

The demonstrated low levels of soluble peroxidase activity in very young seedlings (Fig. 1) suggests that young seedlings may not contain adequate amounts of certain defence-related peroxidase isoforms. Previous studies demonstrated that only velvetleaf seedlings in the cotyledonary stage were killed by infection with *C. coccodes* [27]. This concept has important implications for biological control strategies as it defines a developmental window of maximum weed susceptibility to the biological control agent. Previous reports demonstrated that lesions appeared sooner and seedling mortality increased when both TDZ and *C. coccodes* were applied simultaneously [26]. In this study, although TDZ did not significantly diminish the guaiacol-reactive peroxidase activity induced by *C. coccodes* (Fig. 1), a consistent reduction in soluble peroxidase activity was noted on day 13 post-treatment. These data suggest that TDZ may impair a peroxidase-mediated defence response.

We propose that the anionic isoperoxidases may be associated with the synergistic interaction between TDZ and *C. coccodes* in reducing velvetleaf growth. The combined treatment of velvetleaf with TDZ and *C. coccodes* clearly enhanced three anionic isoforms over control levels within 24 h of treatment (Fig. 3). In contrast, either treatment alone enhanced only a subset of these three isoforms, and enhancement was delayed until the sixth day following treatment (Fig. 3). The simultaneous enhancement of all three isoforms coupled with the reduced lag time may account for the synergistic effect of TDZ and *C. coccodes* treatments on the inhibition of velvetleaf growth.

We have presented data which indicate that TDZ and *C. coccodes* can affect activity and/or expression of specific peroxidase isoforms. The use of two substrates which differ in peroxidase specificity enabled us to correlate the activity of different peroxidase isoforms with distinct growth and defence related events.

TDZ is a kinetin analogue and potent growth regulator. Kinetin, and other growth regulators can alter the activity and isoform pattern of several enzymes, including peroxidase, in *Phaseolus* cultures [2]. Peroxidases have been widely implicated in the growth, development, and pathogen defence mechanisms of higher plants [8, 27]. Application of growth regulators that alter peroxidase activity and isoform expression may have potential undesirable effects on plant growth and development and may increase the susceptibility of plants to natural pathogens through the impairment of defence mechanisms. Considering the potential impact of cytokinin like growth regulators such as TDZ on the growth and pathogen defence mechanisms of crop

plants in addition to the target weeds, studies should be conducted on both crop plants and target weeds before this weed control strategy is applied in the field.

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