

## Peroxidase, polyphenoloxidase, and shikimate dehydrogenase isozymes in relation to tissue type, maturity and pathogen induction of watermelon seedlings

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Isozymes thought to be involved in disease resistance and maturation were investigated in watermelon tissues [*Citrullus lanatus* (Thunb.) Matsum and Nakai] by isoelectric focusing in polyacrylamide gels (IEF). Peroxidase (PER) (EC 1.11.1.7), polyphenoloxidase (PPO) (EC 1.10.3.2), and shikimate dehydrogenase (SKDH) (EC 1.1.1.25), displayed tissue-specific isozymes. Root and cotyledon tissue expressed the most PER isozymes with seven each. All tissue examined contained three PER isozymes between pI 6.5 and 5.5. Spectrophotometric PER analysis showed that total PER activity increased as tissues matured. Root and stem tissue contained the highest PER specific activity, followed by cotyledon, first true leaf and second true leaf. Additional PER isozymes (pI = 3.5, 4.5 and 5.2) were detected as the stem, cotyledon, and leaf tissue aged. When seedlings were inoculated (induced) with an avirulent race of *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *niveum* (E.F.Sm.) W. C. Snyder and H. N. Hans, pI 3.5 and 4.5 PER isozymes were enhanced. After induction, PPO displayed various isozyme patterns and SKDH did not express isozyme polymorphisms. Similar PER isozymes (pI = 3.5 and 4.5) were detected in maturing cotyledon and stem tissue and leaf tissue of root-induced plants, suggesting that PER isozymes enhanced by an avirulent pathogen are also enhanced by maturation.

*Additional key words.* – IEF-PAGE, disease resistance, *Fusarium* wilt, induced resistance, *Citrullus lanatus*.

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**Abbreviations.** IEF, isoelectric focusing; pI, isoelectric point; PAGE, polyacrylamide gel electrophoresis; PER, peroxidase; PPO, polyphenoloxidase; SKDH, shikimate dehydrogenase.

## INTRODUCTION

The shikimate pathway is an important intermediary metabolic pathway in plants leading to the synthesis of aromatic amino acids, indole acetic acid (auxins), cinnamic acids, (precursors to the phenylpropanoid phytoalexins), coumarins, and lignins. Many of these compounds, particularly dihydroxyphenylalanine (DOPA) are associated with senescence or disease resistance mechanisms (Kosuge, 1969) and their synthesis is a function of plant age and tissue location (Ribereau-Gayon, 1972). Several enzymes involved in the shikimate pathway increase in activity with hypersensitive or incompatible disease responses (Cramer *et al.*, 1985). These enzymes include, among others, phenylalanine ammonia lyase (Montalbini and Ragge, 1974), chalcone synthase (Cramer *et al.*, 1985), chalcone isomerase (Cramer *et al.*, 1985), peroxidase (Reuveni and Bothma, 1985), polyphenoloxidase (Bashan *et al.*, 1985), and shikimate dehydrogenase (Montalbini and Ragge, 1974). Hammerschmidt and Kuc (1982), observed that peroxidase isozymes are systemically enhanced in cucumber with resistance induced against *Colletotrichum lagenarium* (Pass.) Ell. and Hals. by a previous infection with the same fungus. Smith and Hammerschmidt (1988) purified acidic peroxidases from watermelon, muskmelon, and cucumber that were induced with *C. lagenarium* and found that the acidic PER were antigenically and electrophoretically similar among the cucurbits. Pathogen-induced PER activity has also been shown to increase in tomato (Kuc, 1982), sugarbeet (Rautelo and Payne, 1970), muskmelon (Reuveni and Bothma, 1985) tobacco (Parent *et al.*, 1985), and other crops (Mayer and Harel, 1979) after infection. Increase in PER activity has been correlated with lignification (Ride, 1975), pathogenesis-related proteins (Parent *et al.*, 1985), hydroxyproline-rich glycoproteins (Hammerschmidt *et al.*, 1984), and suberization (Mohan and Kolattukudy, 1990). Whether these isozyme activities are related directly to pathogen activity or indirectly as a host response to damage is unknown. Recently, Lagrimini *et al.* (1990) showed that a 10-fold expression of an anionic PER in tobacco resulted in wilting at the onset of flowering.

Previously, we have found that when avirulent isolates of *Fusarium oxysporum* f. sp. *niveum*, the causal agent of Fusarium wilt of watermelon, are

applied to the roots, they can protect watermelon from a virulent *F. oxysporum* f. sp. *niveum* isolate (Biles and Martyn, 1989; Martyn *et al.*, 1991). This same root-induction also resulted in a systemically-induced resistance to *C. lagenarium* in leaves (Biles and Martyn, 1989).

As part of an ongoing research program on Fusarium wilt of watermelon, three enzymes believed to be involved in disease resistance, were examined utilizing IEF-PAGE; SKDH, PPO, and PER. Isozymes of each were examined in relation to tissue type, tissue maturation, and inoculation with avirulent and virulent races of *F. oxysporum* f. sp. *niveum*. The objective of this study was to determine if these physiological and pathological influences stimulated differential isozymes that may be useful as biochemical markers for the induced resistance response.

## MATERIALS AND METHODS

**Inoculation technique.** The cotyledon, stem, and root and each existing and newly emerging leaf of 7, 11, 13, and 24 day old watermelon plants was examined. Three-week-old seedlings were inoculated as previously described (Biles and Martyn, 1989). In brief, seedlings were grown in polystyrene foam trays ( $1 \times 1 \text{ cm}^2$  cells) with drain holes which allowed the roots to grow through into a potting medium. At the time of inoculation, the roots were trimmed to approximately 5 cm in length and placed in 250 ml of the inoculum treatment solution. All inocula were adjusted to a concentration of  $1 \times 10^6$  microconidia  $\text{ml}^{-1}$ . The treatments were: (a) a water control; (b) *Fusarium oxysporum* f. sp. *cucumerinum* J. H. Owen (*F. o. cucumerinum*) followed 48 h later by *F. o. f. sp. niveum* race 2. (*F. o. niveum*-2); (c) *F. o. f. sp. niveum* race 0 (*F. o. niveum*-0) followed by *F. o. niveum*-2; (d) *F. o. niveum*-2 followed by water; and (e) water followed by *F. o. niveum*-2. Fungal cultures were maintained and increased as previously described (Martyn, 1987; McKeen and Wensley, 1961). At 6 and 48 h after induction (the first inoculation), leaf tissue was collected from three plants for protein extraction and electrophoresis. In order to verify penetration of the roots by the pathogen, roots from plants used for leaf tissue were excised at the time samples were taken and placed in 5% NaClO for 2 min, rinsed in sterile distilled water, and placed on Komada's (Komada, 1975) medium for reisolation of *F. oxysporum*. In all experiments, *F. oxysporum* was detected growing from the inoculated root tissue after 48 to 72 h.

**Plant material and growing conditions.** Watermelon cultivars used were Black Diamond (Abbott and Cobb

Seed Co.), and Calhoun Gray (Twilley Seed Co.). Black Diamond is susceptible to all races of *F. oxysporum* f. sp. *niveum*. Calhoun Gray is resistant to race 0 and 1 but susceptible to race 2 (Martyn, 1987). Seedlings were grown in styrofoam Speedling® trays (Speedling Inc., Sun City, FL) under fluorescent Grolux (875  $\mu\text{E m}^{-2} \text{s}^{-1}$ ) at 25 to 30°C and fertilized weekly with Peters® solution (20-20-20, NPK) at the manufacturer's recommended rate. Later experiments utilized greenhouse grown watermelon seedlings for spectrophotometric analysis (fig. 3 and 4). Plants were grown in potting soil and watered daily as needed.

**Protein extraction and gel electrophoresis.** Watermelon tissues were excised from the plant (0.5 g) macerated in cold Carlson's modified buffer (0.1 M Tris, 0.1 M KCl, 0.04 M 2-mercaptoethanol, 0.1 M sucrose; pH 7.5) with 50 mg insoluble polyvinyl-polypyrrolidone (PVPP-Sigma No. P-6755) and quartz sand, using a cold mortar and pestle (4°C), and centrifuged (10,000  $\times g$ ). Horizontal slab IEF using 5% polyacrylamide gels (PAGE) was used to detect PER, PPO, and SKDH isozymes. Initial PAGE runs were conducted for each treatment using an ampholine range of pH 3-10. Results showed that all detectable isozyme differences between treatments appeared at the anionic end of the gels. Therefore, in all subsequent gel runs, the ampholine range was narrowed to 3-7 (Servalyte) in order to optimize resolution and separation of the acidic isozymes. The gel apparatus was kept at 7°C during the course of each run. A 1 h pre-run was performed before protein samples were applied. Samples (6  $\mu\text{g}$  protein/sample) were run from cathode to anode at constant power (1 W) for 12-14 h according to the procedure of Benedettelli and Hart (1988). After 12 h, the pH range of the gels was measured using a Corning pH/ion meter 150. The gels were removed from the bed and equilibrated in 0.2 M Tris-HCl buffer (pH 8.5) for 15 min. IEF was also performed using the Pharmacia PhastSystem® and precast Pharmacia® mini-gels. One  $\mu\text{l}$  samples were spotted onto the middle of the IEF gel and protein separation was conducted with a pre-run consisting of 2000 volts (V), 25 milliamps (MA), and 3.5 watts (W) for 75 accumulated volt hours (AVh), a loading run consisting of 200 V, 2.5 MA, and 3.5 W for 15 AVh, and a separation phase consisting of 2000 V, 2.5 MA, and 3.5 W for 410 AVh. All electrophoretic techniques conformed to those recommended by the manufacturer (Pharmacia, NJ). Estimates of isoelectric point (pI) were derived with Sigma IEF markers (Kit No. IEF-MI). PER gels were stained using benzidine dihydrochloride and peroxide as substrates (Brewer and Sing, 1970). PPO gels were stained with catechol and *p*-phenylenediamine as substrates (Czaninski and Catesson, 1972). SKDH gels were stained according to the procedure of Benedettelli and Hart (1988) using shikimic acid as the substrate.

**Peroxidase activity.** Watermelon tissue was prepared as described above and these samples were analyzed for total peroxidase activity. Enzyme activity was measured spectrophotometrically 30 s after 25  $\mu\text{l}$  of sample was added to 1 ml of the substrate (10 mM guaiacol and 10 mM H<sub>2</sub>O<sub>2</sub>). Specific activity was determined as the absorbance per minute per mg of protein at optical density 470 nm. Protein was quantified using the Bradford protein assay (Bradford, 1976). Each experiment was repeated at least twice. One representative experimental data set is presented in the figures.

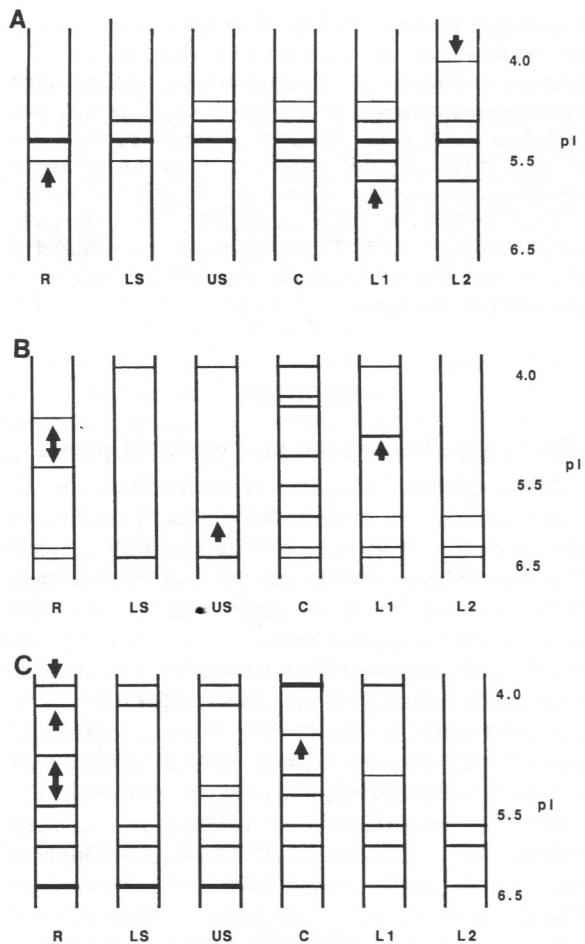
## RESULTS

### Tissue-specific isozymes in 3-week-old plants

Tissue-specific isozymes were detected for all three enzymes in 3-week-old plants. Two SKDH isozymes (fig. 1 A) were detected in the root with the approximate pI=5.0 and 5.5. All other tissues showed at least three isozymes with the first leaf (L1) having the highest number of SKDH isozymes (five). One isozyme (pI=5.6) was present only in leaf tissue (arrow), while another (pI=4.0) was observed only in the newest emerged leaf (L2) (arrow). An isozyme with a pI=5.5 (arrow) was present in all tissue types except the newest leaf.

PPO isozymes showed a different pattern than SKDH (fig. 1 B). The cotyledons had the highest number of isozymes (eight) while the lower stem had the fewest (two). Two isozymes with pI=4.5 and 5.0 (arrows) were unique to root tissue, while one isozyme (pI=5.8) was common to all above-ground tissue except the lower stem (arrow). Leaf tissue displayed a unique isozyme with pI=4.7 (arrow) that, like SKDH, appeared to be developmentally related, since it was present in the first true leaf but not in the newly-emerging leaf. All above-ground tissue, except the newest leaf, shared a common isozyme at pI=4.0. All tissue shared a common isozyme with pI=6.4 suggesting a more constitutive function for this isozyme.

PER isozymes (fig. 1 C) followed a similar pattern as that observed for PPO isozymes, in that photosynthesizing tissue showed an increased number of PER isozymes as the tissue aged. Cotyledon tissue had the greatest number of PER isozymes with seven, while the newly emerging leaf had the lowest number of isozymes (three). All tissues shared three isozymes located at pI=6.4, 5.8, and 5.6, while all tissue except the newly-emerging leaf had a common band at pI=4.0.



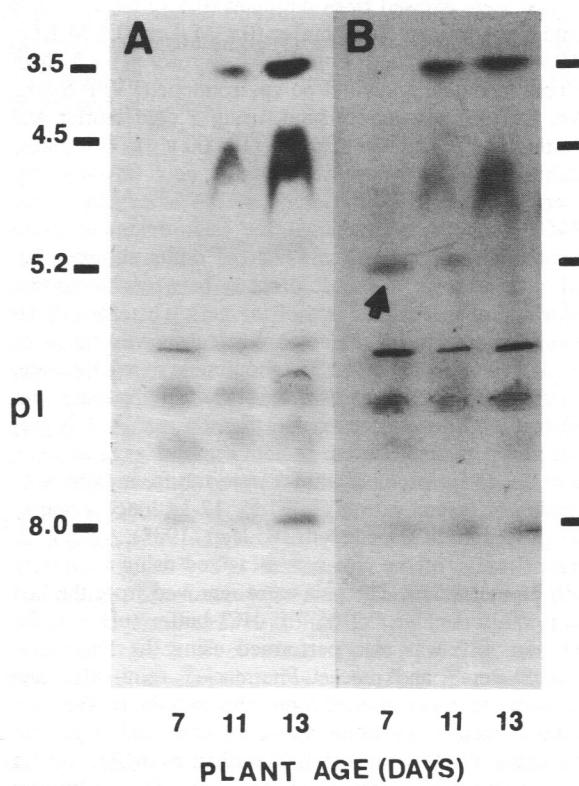
**Figure 1.** Diagrammatic representation of IEF-PAGE gels showing isozyme patterns of three enzymes from different watermelon plant tissues. A, Shikimate dehydrogenase; B, polyphenoloxidase; C, peroxidase. Line thickness is representative of actual band density. Arrows indicate isozymes that were unique to specific tissues. R, root; LS, lower stem; US, upper stem; C, cotyledon; L1, first true leaf; L2, second true leaf. Tissue types were of different ontological ages but all were from 3-week-old plants.

(arrow). Root and stem tissue both showed a PPO isozyme at  $pI=4.4$  (arrow). Roots had a greater number of PPO isozymes (seven), than PPO (four).

#### Tissue-specific isozymes in developing plants

Plant tissues also were sampled at 7, 11, 13, and 24 days after planting. With SKDH, there were no differences in isozymes in cotyledon, leaf, and stem tissue. Each had four similar isozymes at  $pI=4.9$  to 5.5. The newly emerging leaf however, showed an isozyme at  $pI=4.0$  not present in older tissue (data not shown). As the leaf tissue matured, the isozyme patterns became identical. In contrast to

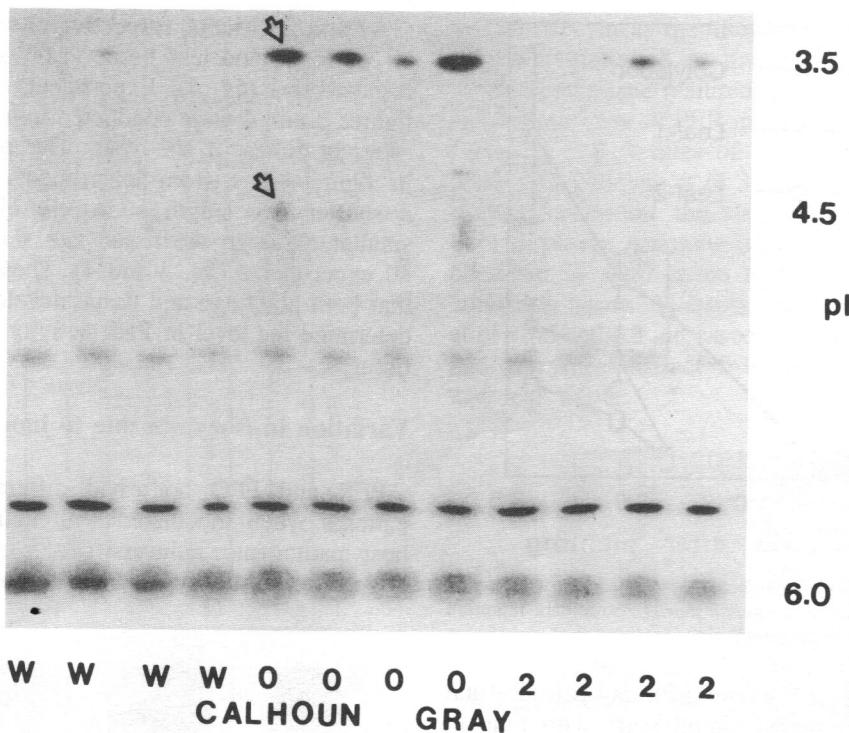
SKDH isozymes, the number of PPO and PER isozymes increased in the photosynthetic tissues as the plant tissue matured. Figure 2 compares the isozyme patterns of PER in cotyledon (fig. 2 A) and stem (fig. 2 B) tissues of 7-, 11-, and 13-day-old plants. An isozyme at  $pI=3.5$  and 4.5 was observed in both cotyledon and stem tissue of 11 and 13 day-old plants. A unique isozyme with  $pI=5.2$  was observed in stem tissue that was not present in cotyledon tissue. As the leaf matured the isozymes at  $pI=3.5$  and 4.5 increased in intensity. Root tissue had isozymes distinct from the cotyledon, stem, and leaf tissue.



**Figure 2.** Peroxidase IEF-PAGE gel of cotyledon (A) and stem tissue (B) of a developing watermelon plant. First, second, and third lanes correspond to tissue from 7-, 11- and 13-day-old plants, respectively. Note the absence of two isozymes ( $pI=3.5$  and 4.5) in both cotyledon and stem at 7 days that were present after 11 days. Arrow indicates a peroxidase isozyme in stem tissue at 7 and 11 days that was not observed in cotyledon tissue. Isoelectric focusing range was  $pI=3.5-8.0$ .

#### Peroxidase activity in developing plants

In separate experiments, total specific activity of PER in different plant tissues was measured



**Figure 6.** Peroxidase isozyme patterns from Calhoun Gray watermelon leaf tissue 6 h after inoculation of roots. Treatments were water (W), avirulent *F. o. niveum* race 0 (0), or virulent *F. o. niveum* race 2 (2). Arrows indicate two enhanced isozymes at pI 3.5 and 4.5 in the avirulent race 0 treatment. Each lane represents one plant. Isoelectric focusing range was pI 4-6.

were consistently the same whether inoculated or uninoculated. In Calhoun Gray watermelon leaves 24 h after inoculation, the water treatment was observed to have the highest number of PPO isozymes and the greatest intensity (fig. 5). However, the *F. o. niveum*-0 treatment had an enhanced isozyme at pI 4.3. The race 2 treatment was missing several isozymes, indicating a reduced PPO activity. The banding patterns between experiments were often variable and inconsistent for PPO.

Differential PER isozymes were observed at 6 and 48 h after inoculation with *F. o. cucumerinum*, *F. o. niveum*-0, and *F. o. niveum*-2 (fig. 6). In the cultivar Calhoun Gray (resistant to race 0 but susceptible to race 2), differential PER isozymes at pI 3.5 and 4.5 were observed as early as 6 h after induction with the avirulent *F. o. niveum*-0 (fig. 6). The cultivar Black Diamond (susceptible to all races of *F. o. niveum*) also displayed enhanced isozymes after inoculation by a non-pathogenic *F. o. cucumerinum* strain (data not shown).

## DISCUSSION

The expression of SKDH isozymes in watermelons appears relatively uniform and stable throughout plant development, which is consistent with the fact that SKDH is associated with chloroplast development (Fielder and Schlutz, 1985). A distinct SKDH isozyme of low pI in newly-emerging leaf may be associated with undeveloped or non-functional chloroplasts. SKDH produces the same isozymes in response to *F. o. niveum* infection, suggesting that the gene product in response to infection is the same as occurs in normal metabolism.

The number of PPO and PER isozymes increased in photosynthetic tissue with plant age. PER isozymes have been studied extensively as a model for hormonal control and physiological processes of the plant (Gaspar *et al.*, 1982) and appear to be compartmentalized to perform specific functions (Abeles *et al.*, 1989 *a*). Tissue specificity of tobacco PER isozymes was also shown by Lagrimini and Rosthstein (1987). Variation among PPO and PER

isozymes in the different tissues (fig. 1) suggest that these isozymes may carry out different functions in the different organs of the watermelon plant.

PER activity can be stimulated by many factors, including heat stress (Stermer and Hammerschmidt, 1985), drought (Smirnoff and Colombe, 1988), disease (Smith and Hammerschmidt, 1988), wounding (Svalheim and Robertsen, 1990), and ethylene (Abeles *et al.*, 1989 *b*; Biles *et al.*, 1990). Abeles *et al.* (1989 *b*) observed an ethylene-induced cationic and anionic PER in several members of the cucurbitaceae family. Our results showed enhanced anionic PER isozymes upon aging (fig. 2) of young root and stem tissue from 7 to 13 days. Watermelon anionic PER isozymes were also enhanced when treated with ethylene suggesting that anionic PER may play a role in tissue maturation (Abeles *et al.*, 1989 *b*).

PER anionic isozymes have been shown to be enhanced by pathogen infection in a resistant cultivar earlier than in a susceptible cultivar in several crops (Flott *et al.*, 1989; Smith and Hammerschmidt, 1988). Our results show that virulent *F. o. niveum* races, as well as a strain of the cucumber with pathogen (*F. o. cucumerinum*), enhance at least two anionic PER isozymes (fig. 6). These *F. o. niveum* races also enhanced resistance to virulent races of *F. o. niveum* and the anthracnose pathogen, *Colletotrichum lagenarium* (Biles and Martyn, 1989). The same PER mechanism activated in other stressed curcurbits appears to be "triggered" in watermelons when induced with an avirulent isolate of *F. o. niveum*. Similar anionic peroxidases were also observed in aging tissue in our study. The fact that these enzymes show similarities in their isozymes in both healthy, maturing tissue, and during an incompatible disease reaction suggests similar or dual roles for these enzymes. Stress caused by several factors mentioned appears to stimulate and enhance these PER isozymes that may have a constitutive function in development. The function that these stress-enhanced peroxidase isozymes serve is still questionable (Abeles *et al.*, 1989 *b*).

In conclusion, SKDH and PPO are not acceptable markers for the induced-resistance response in the *Fusarium*-watermelon wilt system. However, anionic peroxidases observed in the study, are stimulated early in the host-pathogen interaction, and could be useful markers when investigating the induced-resistance response.

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