Arboriculture & Urban Forestry 2010. 36(1): 41–46

Effect of Systemic Inducing Resistance and Biostimulant Materials on Apple Scab Using a Detached Leaf Bioassay

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Abstract. A detached leaf bioassay was used to evaluate several systemic inducing resistance agents, a range of biostimulant products and a conventional triazole fungicide (myclobutanil) on apple scab (*Venturia inaequalis*) development under laboratory conditions. None of the biostimulant products (seaweed extract, betaine, molasses, humic acid, yucca extract, and plant hormone/vitamin complex) evaluated in this study inhibited germination of apple scab conidia, subsequent formation of appressoria or reduced leaf scab severity compared to water treated controls. All SIR agents used in this investigation (potassium phosphonate, potassium phosphite, harpin protein, salicylic acid, salicylic acid derivative) inhibited germination of apple scab conidia, subsequent formation of appressoria and reduced leaf scab severity. The synthetic fungicide myclobutanil resulted in the greatest levels of germination inhibition, reduced appressorium development and leaf scab severity. Results suggest application of an appropriate SIR product may provide a useful addition to existing methods of apple scab management; however, use of biostimulants as scab protectant compounds appears limited.

Key Words. Fungicides; Integrated Disease Management; Pathogen Control; Plant Health Care; Urban Landscapes.

Apple scab caused by *Venturia inaequalis* (Cooke) G. Wint., is one of the most serious diseases of ornamental and fruiting apples (MacHardy 1996). Presently, this disease is controlled mainly by synthetic fungicides applied frequently throughout the growing season (Holb et al. 2005). Identification of fungicide insensitive *V. inaequalis* isolates emphasise the need to evaluate nonfungicidal approaches to scab control (Stanis and Jones 1985; Schnabel and Parisi 1997). Genetically inherent constitutive and inducible defence responses protect trees against insect and pathogen attack (Krokene et al. 2008). Inducible resistance mechanisms such as systemic induced resistance (SIR) can be acquired by exposing plants to organic and/or synthetic compounds such as inorganic potassium and phosphate salts, compost water extracts, low molecular weight proteins, and unsaturated fatty acids (Percival 2001). Developments in plant protection technology have led to the commercial availability of a range of SIR products that have been shown to reduce disease severity in many economically important grasses, crops, and woody plants. These SIR products may offer a potential environmentally benign alternative to synthetic fungicides (Percival and Haynes 2008). These compounds have no direct action on pathogens and are generally of lower mammalian toxicity than synthetic fungicides. Inoculations with the pathogen following treatment with a SIR product fail to cause disease, or the degree of disease severity is reduced (Van Loon et al. 2006). Among the SIR elicitors, harpin protein (Messenger) benzothiadiazole (Bion), potassium phosphite (Agri-Fos), potassium phosphonate (Phytogard), salicyclic acid (DSP), salicylic acid analogs (Rigel), and Probanazole (Oryzemate®), are well-known and have shown to successfully reduce severity of fungal bacterial and viral diseases (Kato et al. 1984; Kessmann et al. 1994; Gorlach et al. 1996; Bécot et al. 2000; Bokshi et al. 2003).

Products sold as biostimulants differ from traditional N:P:K fertilizers in that their active ingredient consists of a range of organic compounds such as plant hormones, humic acids, marine algae extracts, sea kelp, vitamins, and other chemicals that vary according to the manufacturer (Fraser and Percival 2003; Barnes and Percival 2006). Most manufacturers claim biostimulants possess wide activity against a broad range of pathogenic viral, bacterial, and fungal diseases and in addition enhance resistance to insect attack (Mandops 2009; Bio-Plex 2009; Maxicorp 2009). There are, however, few independent scientific studies to support these claims (Grubinger 2005; Portillo et al. 2007).

A laboratory based system for the rapid evaluation of resistance among apple genotypes and determining pathogenesis of *V. inaequalis* isolates was developed using a detached leaf bioassay (Yepes and Aldwinckle 1993a; Yepes and Aldwinckle 1993b). This detached leaf bioassay has been used to successfully evaluate the efficacy of several film forming polymers on apple scab development and suppression. Polymers displaying the greatest degree of scab suppression were carried forward for large scale field-testing, with the degree of scab control shown under field-testing reflecting those obtained under laboratory conditions (Percival and Boyle 2009). Consequently, studies to date indicate adoption of this detached leaf bioassay provides a means of identifying scab protectant properties that commercially available products may, or may not possess.

Aims of this study were to evaluate the efficacy of five SIR and seven biostimulant products against apple scab using a detached leaf bioassay under laboratory conditions.

MATERIALS AND METHODS

Plant Material and Product Treatment

The apple trial site consisted of a 0.75 ha (1.9 acres) block of apple (*Malus* × *domestica* cultivar (cv.) Golden Delicious inter-

spersed with individual trees of *Malus* × *domestica* cv. Red Delicious and Gala as pollinators. Golden Delicious was chosen for experimental purposes due to its sensitivity to apple scab infection. Planting distances were based on 2 m x 2 m (6.6 ft x 6.6 ft) spacing. The trees were planted in 2003 and trained under the central-leader system to an average height of 1.5 m ± 0.15 m (5 ft ± 0.5 ft), with mean trunk diameters of 10 cm ± 1.2 cm (4 in ± 0.5 in) at 45 cm (18 in) above the soil level. The trial site was located at the University of Reading Shinfield Experimental Site, University of Reading, Berkshire, UK (51°43 N, -1°08W). The soil was a sandy loam containing 4%–6% organic matter, pH of 6.2, available P, K, Mg, Na, and Ca were 52.0, 659.1, 175.2, 49.4, and 2188 mg liter (0.0001, 0.005, 0.002, 0.0001, 0.03 oz per gallon), respectively. Five trees were sprayed until runoff with each product at two concentrations (Tables 1 and 2), 28 days after leaf flush (mid-May), a time when leaf material shows maximum photosynthetic performance (Kitao et al. 1998) with no visible symptoms of scab development. The lowest concentration used in Table 2 is based on manufacturer's recommended rate. Double concentration was also tested for each product. Spraying trees were left for 10 days to permit absorption and uptake of each product. By Day 10, twelve fully-expanded leaves per tree were excised from actively growing shoots and all leaf material was prepared within two hours of collection.

Detached Leaf Protocol and Scab Evaluation

Leaves were surface sterilized by immersing in 1% sodium hypochlorite for 30 seconds and then rinsed in sterile distilled water for one minute to remove sodium hypochlorite residues prior to drying on Whatman filter paper (Muhammed et al. 1996). Leaves were then placed abaxial surface down in plastic petridishes lined with moist (sterile distilled water) Whatman filter paper. Five plates with 12 detached leaves per plate (60 leaves per treatment) were inoculated by spraying with an axenic conidial suspension that included a mixture of races 1-5 of *V. inaequalis*. The fungus was grown in wick cultures on 4% malt extract at 18°C (64°F) in the dark, and conidia were collected after washing each wick culture with 10 ml (0.33 fl oz) sterile distilled water, centrifuged (2000 g, 5 min), and re-suspended in distilled water until a suspension of 10^6 conidia ml (0.033 fl oz) was obtained. After inoculation, all plates were sealed with a thin polythene film (Parafilm) permeable to air but not water and incubated in a growth chamber at 19°C ± 1°C (66°F ± 34°F), 16 hours light/8 hours dark photoperiod from white fluorescent tubes at 40μmol m^2 s light intensity (Yepes and Aldwinckle 1993a).

At Day 5, post inoculation the percentage of conidia that had germinated and the percentage that had formed appressoria were determined on 100 spores from 20 leaves, 5 spores per leaf (Yepes and Aldwinckle 1993b). Leaves were decolorized overnight by immersing in 99% cold methanol and stained with periodic acid-basic fuchsin. Whole leaves were mounted on glass slides in glycerol and examined by light microscopy. The remaining forty leaves per treatment were assessed at Day 35 after inoculation using a leaf scab severity rating on the following scale: 0 = No scab observed; 1 = less than 5% of leaf area affected; 2 = 5%–20% of leaf area affected with some yellowing; 3 = 21%–50% of leaf area affected, significant leaf yellowing; 4 = 51%–80% of leaf area leaves affected, severe leaf yellowing; 5 = 81%–100% of leaf area with complete leaf yellowing. All laboratory experiments took place in 2007 and were repeated in 2008. Leaf scab severity ratings used in this study was based on UK and Ireland market standards for fungicide evaluation of scab control (Butt et al. 1990; Swait and Butt 1990). Scab severity ratings were undertaken by three independent BASIS (British Agrochemical Standards Inspection Scheme) qualified crop protection specialists based at Reading University in Reading, UK.

Statistical Methods

All data was analyzed using ANOVA and the differences between means were determined using Tukey *w* procedure (P = 0.05) using the Genstat for Windows program 8th Edition VSN International. Back transformed pathogen severity values are presented here to ease interpretation of these data. Likewise, an arcsine transformation was applied to percentage data before statistical analysis to ensure normality of data. The 2007 and 2008 data sets were not different when compared using a t-test, therefore, values presented represent pooled data for both years.

Table 1. Selected SIR and biostimulant products evaluated for the control of *Venturia inaequalis* on apple cv. Golden delicious under laboratory conditions.

Product	Active Ingredient	Property	Supplier
Water (control)	-	-	-
PhytoGard	Potassium phosphonate	SIR	United Agri Products Ltd, Alconbury Weston, UK
Messenger	Harpin protein	SIR	EDEN Bioscience Corporation, N. Bothell, Washington, USA
Agri-Fos	Potassium phosphite	SIR	Orion Future Technology Ltd, Henwood House, Henwood, Ashford Kent, UK
DSP	Salicylic acid	SIR	Orion Future Technology Ltd, Henwood House, Henwood, Ashford Kent, UK
Rigel	Salicylic acid derivative	SIR	Orion Future Technology Ltd, Henwood House, Henwood, Ashford Kent, UK
Maxicrop Original	Seaweed extract	Biostimulant	Ciba Sp Maxicrop (UK) Ltd, P.O. Box 6027, Corby, UK
Resistim	Betaine	Biostimulant	Mandops UK Ltd, Eastleigh, Hampshire, UK
Bioplex 12-4-6.	Seaweed + humic acid extract	Biostimulant	United Agri Products Ltd, Alconbury Weston, UK
Fulcrum CRV	Molasses	Biostimulant	Banks Cargill Agriculture Ltd, St Hughs, Lincoln, UK
Redicrop	Natural plant compound	Biostimulant	United Agri Products Ltd, Alconbury Weston, UK
Crop Set	Yucca based material with liquid fermentation products	Biostimulant	United Agri Products Ltd, Alconbury, Weston, UK
Superthrive	Plant hormone/vitamin complex	Biostimulant	Urban Hydroponics, Unit 1, Back Lane, Bolton, UK
Systhane	Myclobutanil	Synthetic fungicide	Syngenta Crop Protection UK Ltd, Whittlesford, Cambridge, UK

SIR = Systemic Induced Resistance

Table 2. Germination of conidia[z], formation of appressoria[y] and scab severity rating[x] on apple leaves of *Malus* × *domestica* cultivar Golden Delicious.[w]

Treatment	Concentration per litre (0.26 gal)	Germination (%) at Day 5	Conidia with appressorium (%) at Day 5	Leaf severity rating at Day 35
Water (control)	-	93±11.0ab	90±8.0a	5.0±0.60a
PhytoGard	5.0 ml (0.17 fl oz)	52±6.0def	59±6.1cd	2.6±0.23de
PhytoGard	10 ml (0.33 fl oz)	36±4.2fg	44±4.7e	2.0±0.20ef
Messenger	3.2 g (0.14 oz)	68±7.3bcde	77±8.5abc	3.2±0.36cd
Messenger	6.4 g (0.28 oz)	58±5.3de	50±4.7de	2.4±0.30def
Agri-Fos	10 ml (0.33 fl oz)	49±4.1ef	70±8.0abc	3.0±0.26cd
Agri-Fos	20 ml (0.66 fl oz)	44±3.8ef	63±7.5bcd	2.7±0.30cde
DSP	3.0 ml (0.10 fl oz)	46±5.0ef	58±4.9cde	2.3±0.30ef
DSP	6.0 ml (0.20 fl oz)	40±5.0fg	63±5.5bcd	2.2±0.38ef
Rigel	3.0 ml (0.10 fl oz)	60±6.1cde	73±5.0abcd	3.3±0.42bcd
Rigel	6.0 ml (0.20 fl oz)	55±5.9de	60±6.1cd	3.0±0.38
Maxicrop Original	10 ml (0.33 fl oz)	90±12.0ab	91±10.2a	4.8±0.52ab
Maxicrop Original	20 ml (0.66 fl oz)	94±10.4ab	92±9.7a	5.0±0.55a
Resistim	10 ml (0.33 fl oz)	88±9.8a	90±7.6a	4.9±0.48a
Resistim	20 ml (0.66 fl oz)	92±13.1ab	90±8.0a	5.0±0.57a
Bioplex	10 ml (0.33 fl oz)	90±10.5ab	93±9.8a	5.0±0.63a
Bioplex	20 ml (0.66 fl oz)	86±8.8abc	87±8.9a	4.6±0.5.7ab
Fulcrum CRV	10 ml (0.33 fl oz)	96±13.4a	93±10.7a	5.0±0.60a
Fulcrum CRV	20 ml (0.66 fl oz)	94±12.1ab	90±9.0a	4.9±0.55a
Redicrop	10 ml (0.33 fl oz)	88±11.1ab	86±9.9a	5.0±0.61a
Redicrop	20 ml (0.66 fl oz)	92±12.5ab	90±9.0a	5.0±0.60a
Crop Set	10 ml (0.33 fl oz)	95±14.5a	93±10.2a	5.0±0.63a
Crop Set	20 ml (0.66 fl oz)	93±11.0ab	90±8.7a	5.0±0.62a
Superthrive	0.25 ml (0.008 fl oz)	80±8.9abc	81±8.3ab	4.4±0.44ab
Superthrive	0.50 ml (0.16 fl oz)	77±8.0abcd	80±8.0abc	4.0±0.39abc
Systhane	0.3 ml (0.01 fl oz)	27±2.6fg	35±4.2e	1.3±0.21ef
Systhane	0.6 ml (0.02 fl oz)	15±1.7g	31±4.0e	1.0±0.16f

[z] As % of spores.
[y] As % of germinated spores.
[x] Detached leaf scab severity index: scale 0 = No scab observed; 1 = less than 5% of leaf area affected; 2 = 5%–20% of leaf area affected with some yellowing; 3 = 21%–50% of leaf area affected, significant leaf yellowing; 4 = 51%–80% of leaf area leaves affected, severe leaf yellowing; 5 = 81%–100% of leaf area with complete leaf yellowing.
[w] Data represents pooled mean of 2007 and 2008 studies except Rigel and Superthrive that were evaluated in 2008 only. Mean ± standard deviation of 80 leaves (germination of conidia, formation of appressoria) and 160 leaves scab severity rating. Lower case letters indicate significant differences between means for each evaluation date by Tukey highly significance test ($P = 0.05$). The lowest concentration used in Table 2 is based on manufacturer's recommended rate (single strength). The other concentration tested represents a double strength product application.

RESULTS AND DISCUSSION

Irrespective of concentration applied, none of the biostimulants products (Maxicrop Original, Resistim, Bioplex 12-4-6, Fulcrum CV, Redicrop, Crop Set, Superthrive) evaluated in this study inhibited germination of apple scab conidia, subsequent formation of appressoria or reduced leaf scab severity. In all cases, values recorded were statistically comparable with water based controls (Table 2). In support of this, Portillo et al. (2007) found little efficacy of a range of biostimulant active ingredients when evaluated *in vitro* against *Phytophthora infestans* of tomato. Likewise, data from field trials also seems to indicate little efficacy of products containing plant extracts against a range of pathogenic fungi (Grubinger 2005; Chalker-Scott 2005). However, research elsewhere indicated application of Yucca extracts provided control of apple scab comparable to sulphur using apple cv. Jonagold seedlings (Köhl 2006). Despite the apparent robustness of the detached leaf bioassay system used in this study (Yepes and Aldwinckle 1993a; Yepes and Aldwinckle 1993b; Percival and Boyle 2009), it should be emphasized results were obtained under laboratory conditions and may fluctuate from those obtained at the whole plant level.

All SIR agents used in this investigation (potassium phosphonate, potassium phosphite, harpin protein, salicylic acid, salicylic acid derivative) inhibited germination of apple scab conidia by 27% to 61%, subsequent formation of appressoria by 14% to 51%, and reduced leaf scab severity by 36% to 60%. In virtually all cases, the higher concentration of SIR product applied the greater the degree of inhibition was recorded (Table 2). Of the SIR products tested, potassium phosphonate (PhytoGard) applied at 10 ml per liter (0.26 gal) was the most effective inhibitor of germination five days after inoculation and showed the greatest level of inhibition of appressorium development (Table 2). In addition, this product resulted in the lowest leaf scab severity. Of the SIR products tested, harpin protein (Messanger) at 3.2 g (0.14 oz) per liter was the least effective inhibitor of germination and appressorium development and a salicylic acid derivative (Rigel) applied at 3.0 ml (0.1 fl oz) per liter least effective in reducing leaf scab severity (Table 2). With reduced sensitivity to sterol inhibiting fungicides in field isolates of *Venturia inaequalis* identified as early as the mid 1980s, a greater emphasis on alternative pathogen control strategies to extend the effective commercial life of current synthetic fungicides and reduce the risk of resistance development is now advocated (Stanis and Jones 1985; Schnabel and Parisi 1997). Results of this investigation indicated that, under laboratory conditions, all SIR products tested inhibited germination of apple scab conidia, subsequent formation of appressoria and scab severity. Similar effects caused by SIR elicitors on the physiological development of root and foliar diseases to include downy mildew (*Peronospora parasitica*), bar-

ley powdery mildew (*Blumeria graminis* f. sp. hordei), cucumber powdery mildew (*Sphaerotheca fuliginea*), downy mildew (*Bremia lactucae*), rust (*Puccinia sorghi*) charcoal rot (*Macrophomina phaseolina*), have been shown elsewhere (Kessmann et al. 1994; Reuveni et al. 1994; Reuveni et al. 1995; Godard et al. 1999; Bécot et al. 2000; Pajot et al. 2001; Srivastava et al. 2001; Martinelli et al. 2007). These studies suggested the detrimental effects on fungal development were the result of several modes of action to include synthesis of β-1,3-glucanases, chitinases (Busam et al. 1997), cysteine-rich proteins, beta-(1,3)-glucanase and the PR-1 proteins (Anfoka and Buchenauer 1997) by leaf and root tissue in response to SIR elicitors. Other morphological and biochemical changes in SIR protected plants include a significantly faster lignification response, which corresponded with an increase in peroxidase activity (Ajilan and Potter 1992), an accumulation of fungi-toxic β-ionone derivatives (Wyatt and Kuc 1992), induction of lipoxygenase (Staub et al. 1992), antimicrobial fatty acid derivatives (Namai et al. 1993), phenylalanine ammonia-lysase, phytoalexins (Elliston et al. 1977) and hydroxyproline-rich glycoprotein (Raggi 1998). Within conifers inducible defense systems include secondary resin production, synthesis of new phenolics, traumatic resin duct formation and initiation of a wound periderm (Franceschi et al. 2000).

The synthetic fungicide myclobutanil applied at 0.6 ml (0.02 fl oz) per liter resulted in the greatest germination inhibition (84%), reduced appressorium development (67%) and leaf scab severity (80%) recorded in this study (Table 2). Within the UK and Ireland, myclobutanil is fully approved for scab control of ornamental and fruiting apples, identified as possessing protective and curative action and anti-sporulation activity (Anonymous 2008). The antisporulant and fungicidal effectiveness of myclobutanil against scab under laboratory and field conditions has been confirmed by other workers when evaluating fungicides for UK and Ireland marketing purposes (Butt et al. 1990; Swait and Butt 1990). Previous research indicates most commercially available SIR agents are generally less effective and consistent than standard synthetic fungicides for foliar disease control (Agostini et al. 2003; Percival and Haynes 2008). Results of this study support these conclusions with myclobutanil proving to be the optimal treatment in terms of spore germination inhibition, reduced appressorium development and leaf scab severity.

In conclusion, significant reductions in scab severity recorded in this study do give credence to the potential of SIR products as an alternative or compliment to conventional fungicides. As SIR products have no direct effect on pathogens then they are not, at least in the UK, subject to the stringent legislative restrictions that relate to the use and application of conventional pesticides that act by chemical means. Likewise even though induced resistance products are not totally effective for scab control, they may be useful in an integrated program with standard fungicides. Use of biostimulants as scab protectant compounds, however, appears limited based on results of this study.

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TREE FUND
Tree Research & Education Endowment Fund

Acknowledgments. The authors are grateful for funding, in part, from the TREE FUND (Hyland Johns Grant).

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Résumé. Un bio-essais foliaire séparé a été utilisé pour évaluer un certain nombre d'agents pour induire une résistance systémique, soient une variété de bio-stimulants et un fongicide conventionnel de triazole (myclobutanil), contre le développement de la tavelure (*Venturia inaequalis*) sous des conditions de laboratoire. Aucun des bio-stimulants (extrait de plantes aquatiques, betaine, mélasses, acide humique, extrait de yucca, complexe hormonale/vitaminique) évalués dans cette étude n'a inhibé la germination des conidies de la tavelure, la formation subséquente des hyphes ou encore diminué la sévérité de la tavelure comparativement au groupe-témoin traité avec de l'eau. Tous les agents utilisés dans cette étude–phosphonate de potassium, phosphite de potassium, protéine harpine, acide salicylique, dérivé d'acide salicylique–ont inhibé la germination des conidies de la tavelure, la formation subséquente des hyphes et diminué la sévérité de la tavelure. Le fongicide synthétique myclobutanil a produit les meilleurs résultats quant à l'inhibition de la germination, la diminution de la formation des hyphes et la sévérité de la tavelure. Les résultats suggèrent que l'application d'agents appropriés peuvent constituer un complément utile aux méthodes existantes de gestion de la tavelure; cependant, l'utilisation de bio-stimulants comme agent protecteur face à la tavelure apparaît limitée.

Zusammenfassung. An Blättern wurde ein Biotest vorgenommen, um den Einfluss von verschiedenen systemischen Mitteln zur Erhöhung der Resistenz zu bewerten, eine Auswahl von Biostimulanz-Produkten und ein konventionelles Triazol-Fungizid bei der Entwickung von Apfelschorf-Entwicklung unter Laborbedingungen. Keins der Biostimulanz-Produkte (Seetang-Extrakt, Betain, Molasse, Huminsäure, Yucca-Extrakt und Pflanzenhormon/Vitamin-Komplex), die in dieser Studie bewertet wurden, hemmten die Germination von Apfelschorf-Konidien, die subsequente Formation von Apressoria oder reduzierte die Schorfinfektion der Blätter, verglichen mit einer Kontrolluntersuchung, die nur mit Wasser behandelt wurde. Alle SIR-Agentien, die in dieser Studie verwendet wurden (Kaliumphosphonat, Kaliumphosphid, Harpin-Protein, Salicylsäure, Salicylsäure-Derivate) hemmten die Konidienbildung, die subsequente Appressoria-Bildung und reduzierten den Befall der Blätter. Die Ergebnisse verdeutlichen, daß die Applikation von dem richtigen SIR-Produkt eine nützliche Erweiterung der herkömmlichen Methoden zur Apfelschorf-Bekämpfung sein kann, während die Anwendung von Biostimulantien als Schutzstoff ein begrenztes Spektrum hat.

Resumen. Se usó un bioensayo foliar para evaluar la resistencia de varios agentes sistémicos, un rango de productos biolestimulantes y un fungicida convencional triazol (miclobutanil) en la costra foliar del manzano (*Venturia inaequalis*) desarrollado bajo condiciones de laboratorio. Ninguno de los productos bioestimulantes (extracto de algas, betaína, melaza, ácido húmico, extracto de yuca y complejo hormona/vitamina) evaluado en este estudio inhibió la germinación de las conidias de la costra, formación subsecuente de apresoria o reducida severidad de la costra, comparado a controles tratados con agua. El fungicida sintético miclobutanil dio los niveles de inhibición de la germinación más grandes, desarrollo reducido de *apresurium* y severidad de la costra foliar. Los resultados sugieren que la aplicación de un producto SIR apropiado puede proveer una adición útil para métodos existentes de manejo de la costra foliar del manzano; sin embargo, el uso de bioestimulantes, como compuesto, parece limitado.