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# TECHNIQUE FOR QUANTIFYING VIRULENCE OF *FUSARIUM* AND *CYLINDROCARPON* SPP. ON CONIFER GERMINANTS

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## BACKGROUND

*Fusarium* and *Cylindrocarpon* spp. are common root-inhabiting fungi which often colonize cortical root cells of container-grown and bareroot conifer seedlings, sometimes causing disease. There are specific environmental and site conditions which affect seedling infection and subsequent disease severity. These include temperature (Pomerleau 1942; Strobel and Sinclair 1991; Vaartaja and Bumberis 1967), moisture (Pomerleau 1942; Schneider and Pendery 1983; van den Driessche 1963), soil pH (Scher and Baker 1982; Tint 1945b), nutrition (Duda and Sierota 1987; Scher and Baker 1982), and previous crop history (Baayen and others 1989). In both genera, only certain isolates are capable of eliciting seedling disease; these isolates may express specific pathogenicity genes allowing successful infection of hosts and disease production (Bloomberg 1981; Bloomberg and Lock 1972; Matuo and Chiba 1966). This inherent pathogenic quality is often difficult to elucidate because of compounding factors influencing disease expression (Tint 1945a).

Certain species of *Fusarium* and *Cylindrocarpon* cause disease on conifer seedlings more readily than others. Also, within a particular taxon, isolates may be either pathogenic or non-pathogenic, but morphologically indistinguishable (Matuo and Chiba 1966; Tint 1945a). This is particularly true for *F. oxysporum* Schlecht. (Elmer and Stephens 1989; Ho and others 1985); members of this species are extremely diverse genetically, resulting in parasitic and saprophtyic behavior (Bloomberg 1971; Huang and Kuhlman 1990; Smith and Snyder 1975). In addition, cultural morphology may also be diverse and respond to differing culture media and incubation conditions (Brown and Horne 1926; Gerlagh and Blok 1988).

During routine screening of soil and seedling root tissue samples, many *Fusarium* and *Cylindrocarpon* isolates are often obtained. Formerly, isolates commonly associated with disease symptoms, i.e., necrotic lesions on roots manifested by above-ground chlorosis and necrosis of foliage, were usually considered pathogenic (James 1985, 1986b; James and others 1987). Yet most appear morphologically identical to other isolates obtained from either roots of non-diseased seedlings or soil (Booth 1971; Nelson and others 1983).

Several new techniques were devised to address this problem of variable pathogenicity, particularly within the genus *Fusarium*. Such procedures include protein, toxin and immunological analysis (Del Sorbo and others 1993; Desjardins and others 1993; Ho and others 1985; Singh and Hoffmann 1969), vegetative compatibility grouping (Elmer and Stephens 1989; Katan and others 1989; La Mondia and Elmer 1989; Puhalla 1985), and molecular analysis of nucleic acid including restriction fragment length polymorphisms (Coddington and others 1987; Kim and others 1992), random amplified polymorphic DNA (Manulis and others 1994), polymerase chain reaction amplification with probing (Di Pietro and others 1994; Hawthorne and others 1994), direct nucleic acid sequencing (Gordon and Okamoto 1992) and electrophoretic karyotyping (Boehm and others 1994; Fekete and others 1993; Migheli and others 1993). These procedures differentiate test isolates on the basis of genetic variability that may be manifested by protein production, hyphal anastomosis and nuclear exchange, and alterations in nuclear and cytosolic genetic material. Technique refinement is by no means complete and new and improved procedures are being continually developed. In the long run, molecular techniques hold the most promise for easily differentiating fungal isolates on the basis of many characteristics, including pathogenicity (Gordon and Okamoto 1992). Unfortunately, expensive laboratory equipment and technical expertise are essential to routinely conduct molecular tests. Many diagnostic laboratories processing plant and soil samples are not currently capable of conducting such tests and must rely on simpler, less intensive evaluations.

Diverse approaches have been made to rapidly assess pathogenic capabilities of specific *Fusarium* isolates (Vaartaja and Bumberis 1967; Vaartaja and Cram 1956). Most techniques introduce inoculum on-

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lates (Vaartaja and Bumberis 1967; Vaartaja and Cram 1956). Most techniques introduce inoculum onto apparently healthy plants and assess production of disease symptoms (Freeman and Rodriguez 1993). Inoculum types include spores (macroconidia, microcondia, chlamydospores), vegetative mycelia, and artificial inoculum using a particular food base for fungal growth. Tests run for various times depending on crop type evaluated (including stage of crop development) and type of disease symptoms desired (Freeman and Rodriguez 1993).

On conifer seedlings, pathogenicity tests of *Fusarium* spp. often have varying results for many reasons (Edmonds and Heather 1973; Hildebrand 1985; Rathbun-Gravatt 1925). Although root infection readily occurs, production of disease symptoms may be variable, especially on plants several months or years old (Gerdemann and Finley 1951; Huang and Kuhlman 1990; Matuo and Chiba 1966). In some cases, environmental stresses are required to induce disease and symptom production may not be repeatable for particular isolates (Bloomberg 1971; Davis 1963). Because of these problems, a rapid, repeatable procedure to quantify virulence of *Fusarium* and *Cylindrocarpon* isolates on Douglas-fir seedlings was developed. Douglas-fir was chosen as a test species because of its susceptiblity to these fungi, and the prevalence of damping-off and root disease on this species in both container and bareroot nurseries (James 1986a; James and others 1991, 1994).

This pathogenicity test procedure yields consistent results when repeated with the same test isolates of *Fusarium* and *Cylindrocarpon* over several months. Numerical ratings may vary somewhat within a test, but are generally not widely divergent among different tests. The procedure can be completed quickly with results in about two weeks. Unlike other tests, stressing seedlings to induce disease is not required (Bloomberg 1971; Davis 1963). In addition, equipment needs are minimal. One disadvantage is that the test requires daily examination of germinants since the scoring system is based primarily on when disease symptoms become evident. Inoculum preparation may be time-consuming, but once the perlite/cornmeal inoculum is prepared, it will remain viable for a long time.

This pathogenicity test has been used to evaluate virulence of several different groups of *Fusarium* and *Cylindrocarpon* isolates. Examples include analyses of soil-borne isolates of *F. oxysporum* from bareroot nurseries, different isolates of *F. proliferatum* (Matsushima)Nirenberg obtained from healthy and diseased container-grown seedlings, and interactions between pathogenic *Fusarium* species and potential biocontrol agents such as *Gliocladium virens* Miller, Giddens & Foster and *Streptomyces griseoviridis* (strain K61). This test can provide the basis for further greenhouse or field testing of potential biocontrol agents.

The rapid, reliable pathogenicity test described next may be an important supplement to genetic analysis. Genetic differences related to pathogenicity must be confirmed with actual *in vivo* tests to evaluate capability of fungal isolates to elicit disease.

## PATHOGENICITY TEST PROCEDURES

The basic approach is to expose young Douglas-fir germinants to tested fungal inoculum and record production of disease symptoms. Because of past success (James and Gilligan 1994; James and others 1989) cornmeal-perlite inoculum is used. This inoculum is prepared using the techniques of Miles and Wilcoxon (1984). Perlite, an inert, inorganic, siliceous rock of volanic origin commonly used in potting mixtures, is the matrix for fungal growth. 150g of yellow cornmeal are moistened with 300 ml warm 1 percent potato dextrose agar (PDA), to which 75g of perlite are added. The mixture is placed into glass vials (23 ml capacity) to about two-thirds capacity which are then autoclaved for 60 min at 121°C. After cooling, vials are inoculated with about 10 ml spore suspension of the test fungus (produced by adding sterile, distilled water to 14 day-old cultures grown on PDA). Vial caps are left loose to allow aeration. Vials are incubated in the dark for at least 21 days, after which the fungus has thoroughly colonized the perlite/cornmeal mixture. After incubation, inoculum is removed from vials and dried in open petri plates within a cabinet. Inoculum dries within 5-7 days and does not become contaminated with other organisms because the food base is completely colonized by the inoculated fungus. Once dry, inoculum is stored in plastic vials and refrigerated until needed. This type of inoculum has remained viable under refrigeration for at least 2 years.

When developing this pathogenicity technique, various media were evaluated. Perlite, standard peat-vermiculite growing media (used in most commercial forest seedling greenhouse operations), and 50:50 coconut husk-vermiculite medium were examined as a substrate for growing young germinants. Peat-vermiculite formulations vary widely depending on manufacturer, and may result in differences in moisture absorbance that affects germinant root growth, altering exposure to test inoculum. The coconut-vermiculite mixture (Grace/Sierra Horticultural Products, Milpitas, CA) works well because it is consistent and does not change volume when wetted.

Twenty-four vials (23 ml capacity each) are used to test each fungal isolate. Each vial is filled to about two-thirds capacity (about 2.5g) with dried media and autoclaved at 121°C for 60 minutes. Vial lids, if they can be autoclaved, are placed loosely on vials before sterilization. Plastic lids may be sterilized in a bleach/alcohol solution.

Douglas-fir seedlots and standard seed handling procedures minimize variability caused by seed. The selected seedlot should have high germination capacity and energy. Seed is soaked in a 2-part bleach and 3-part water solution for 10 minutes (Wenny and Dumroese 1987), rinsed 48 hours in running tap water, and stratified 21 days at 2-3°C (interior seed sources). After stratification, seed is placed on filter paper moistened with sterile water, in sterile petri plates. Seed is incubated under 12-hour diurnal fluorescent light cycles at about 24°C and monitored daily for germination. Seed is considered germinated when its primary root is at least 3 mm long. At the time of germination, primary roots are checked for rigidity to ensure they are not decayed.

Perlite/cornmeal inoculum is ground to a fine powder with mortar and pestle and 0.05 g of the powder is added to each vial containing dried media. This results in an 1:50 w/w mixture of inoculum to media. Inoculum is distributed throughout the media by shaking. One recently germinated seed (germinant) is placed in each vial with its radicle placed downward into the media. Four ml sterile water is added to each vial with caps replaced loosely to allow aeration. Adding water activates inoculum (Miles and Wilcoxon 1984). At least 20 of the 24 vials must have germinants with normally extending roots for a valid test. Occasionally, a germinant's root will abort or decay because of microorganisms contained within the seed. For each pathogenicity test, one set of 24 control vials made by adding non-inoculated perlite instead of inoculum is evaluated.

Vials containing germinants are incubated at about 24°C on a lab bench or within an incubator, providing fluorescent light for 8-12 hours daily. Each test runs for 14 days. After 3 days, germinants are first checked for disease. During this inspection, germinant roots may need to be reoriented downward into the medium. Germinants are checked for disease daily. Standard post-emergence damping-off occurs when the germinant root is attacked by the test fungus growing just above the groundline (figure 1). Post-emergence root decay occurs when the germinant root is attacked but visible fungal growth at or above the groundline is lacking. In this case, the root is decayed (usually a wet type) or the growing tip is either decayed (brown and flexible - figure 2) or completely sloughed off. After 7 days' incubation, if root decay is suspected because germinants are not growing well or their root has not grown to the bottom of the vial, germinants are carefully removed with forceps and inspected for decay. If decay is lacking, germinants are replaced and the test resumed. Germinants surviving 7 days receive an additional 2 ml sterile water. After 14 days, surviving germinants are examined to determine if their roots have grown to the bottom of the vial. Their roots are also examined for disease symptoms (decay or necrotic lesions). Healthy roots are usually white and actively growing.

A numerical rating system for isolate comparisons awards points based on duration of germinant survival within inoculated vials, occurrence and type of disease, reisolation of inoculated fungal isolate, and primary root growth within the vial (Table 1). The range of possible points is 3-23, with higher point values reflecting less aggression by the tested isolate against the germinant. To convert points to a score in which higher numbers represent greater virulence, a reciprocal rating was devised (Table 2). In this system, the maximum score (all germinants killed within 3 days by the test isolate) is 100 and the minimum score (indicating germinants were uninfected within 14 days) is zero. The average rating for all germinants tested against a particular isolate is used to compare isolates.

There will be some variability in the response of germinants to tested fungal isolates. For example, a few germinants may succumb to the test fungus after a few days, but others may survive longer. A notable exception is very aggressive isolates which quickly kill all germinants. However, after averaging all ratings within the 24 vial test, consistent differences among isolates are usually found.

Because of the wide range of scores possible in this procedure, specific virulence categories (high, medium, low) include scores varying up to 20 points. Isolates considered highly virulent average scores from 80-100; those considered moderately virulent average scores from 60-80, and isolates averaging scores of 40-60 have low virulence. Isolates with average scores below 40 are considered non-pathogenic, even though a few germinants may have been infected and killed by these isolates under test conditions.

Figure 1. Standard post-emergence damping-off of young Douglas-fir germinant inoculated with *Fusarium oxysporum*. Germinant was exposed to test fungus for 7 days.

Figure 2. Tip of Douglas-fir germinant root infected and decayed by *Fusarium oxysporum* after exposure to the fungus for 7 days.

Table 1. Numerical point system for pathogenicity test of Douglas-fir germinants inoculated with fungal test isolates.

| Germinant Survival | Days Survival | Points |
|---|---|---|
| | 3 | 1 |
| | 4 | 2 |
| | 5 | 3 |
| | 6 | 4 |
| | 7 | 5 |
| | 8 | 6 |
| | 9 | 7 |
| | 10 | 8 |
| | 11 | 9 |
| | 12 | 10 |
| | 13 | 11 |
| | 14 | 12 |
| Disease Incidence | Type of Disease* | Points |
| | DO | 1 |
| | WR | 2 |
| | ND | 4 |
| Fungal Reisolation | Reisolation Category** | Points |
| | Pure | 1 |
| | OTHFUS | 2 |
| | None | 4 |
| Radicle Penetration | Category** | Points |
| | BOT | 3 |
| | None | 0 |

* DO = standard post-emergency damping-off with fungal growth on primary root above the groundline. WE = root attacked and diseased with decay or growing tip brown and no longer functional. ND = no disease symptoms.

** Pure = reisolated the inoculated isolate only. OTHFUS = reisolated another *Fusarium* isolate in addition to the inoculated isolate. None = did not reisolate the inoculated isolate (fungi other than *Fusarium* may have colonized the root).

*** BOT = primary root penetrated to the bottom of the vial during the 14-day test. None = radicle did not penetrate to the bottom of the vial.

Table 2. Scoring system for virulence comparisons among fungal test isolates on Douglas-fir germinants.

| Point Total* | Score | Point Total* | Score | Point Total* | Score |
|---|---|---|---|---|---|
| 3 (Min.) | 100 | 10 | 65 | 17 | 30 |
| 4 | 95 | 11 | 60 | 18 | 25 |
| 5 | 90 | 12 | 55 | 19 | 20 |
| 6 | 85 | 14 | 50 | 20 | 15 |
| 7 | 80 | 14 | 45 | 21 | 10 |
| 8 | 75 | 15 | 40 | 22 | 5 |
| 9 | 70 | 16 | 35 | 23 (Max.) | 0 |

* Point system for individual germinants is described in table 1. Scores for all germinants exposed to a particular test isolate are averaged.

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