Screening Shoot Cultures of *Malus* for Cedar-Apple Rust Infection by In Vitro Inoculation

H. JOUNG, Graduate Research Assistant, S. S. KORBAN, Assistant Professor, and R. M. SKIRVIN, Associate Professor, Department of Horticulture, University of Illinois, Urbana 61801

ABSTRACT

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A simple and effective method for screening shoot cultures of *Malus* for resistance to the cedar-apple rust fungus (*Gymnosporangium juniperi-virginianae*) was developed. Shoot-tip cultures of Jonathan apple and Prairiefire crabapple cultivars were inoculated in vitro with a *G. juniperi-virginianae* basidiospore suspension and evaluated for rust symptoms. Jonathan, a susceptible cultivar, developed pycnial lesions of the rust within 10 days of inoculation, whereas Prairiefire, a resistant cultivar, showed no symptoms. No extraneous contamination was observed. In vitro inoculation of adventitious and axillary shoots derived from cotyledonary and embryo axis tissues, respectively, did not detect any somaclonal variation for resistance to the rust fungus.

Additional key words: disease screening

Cultivated apple (*Malus* × *domestica* Borkh.) and crabapple tissues have been successfully grown in vitro (12,13). Tissue cultures have been used in the *Malus* system to study the influence of media, metabolites, and environmental conditions on shoot proliferation (6,13), to induce adventitious shoot formation (4), to induce callus formation, and to isolate protoplasts (2,3). In only a few studies have apple tissue cultures been used successfully to inoculate and screen for disease resistance in vitro. Saad (8) inoculated apple callus with a spore suspension of *Venturia inaequalis* (Cke.) Wint. and observed inhibition of callus growth by this pathogen. Wanstreet (12) developed a system for inoculating crabapples with *Erwinia amylovora* (Burr.) Winslow et al in vitro and observed a high correlation between susceptibility of plants in the orchard and shoot-tip cultures in vitro. In this study, we describe a simple, in vitro inoculation technique for screening shoot cultures of *Malus* for resistance to the cedar-apple rust fungus (*Gymnosporangium juniperi-virginianae* Schw.). Experiments to test the reliability of this method are presented, and a preliminary investigation of its use to study genetic and/or somatic variability is also presented.

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MATERIALS AND METHODS

Shoot cultures. Dormant scion wood (30–40 cm) of Jonathan apple and Prairiefire crabapple were placed in a flask containing distilled water in the laboratory (23 C) to induce bud growth and development. Expanding vegetative buds were excised, surface-sterilized in a 0.5% sodium hypochlorite solution containing 0.1% Tween 20 for 15 min, and then rinsed three times in sterilized distilled water. Shoot tips (5–10 mm) were then placed into culture tubes (25 × 100 mm) containing a shoot proliferation medium (SPM). SPM consisted of high-mineral Murashige and Skoog (MS) salts (4.3 g/L) (7), Staba vitamins (10 ml/L) (11), *myo*-inositol (100 mg/L), ascorbic acid (50 mg/L), benzyladenine (BA) (2 mg/L), naphthalene acetic acid (NAA) (0.1 mg/L), and sucrose (30 g/L). After the pH was adjusted to 5.7 with 0.1 N KOH, Difco-Bacto agar (7 g/L) was added and the medium was autoclaved for 15 min. Cultures were grown in a controlled-temperature and light room at 22 ± 2 C under 16 hr of cool-white fluorescent light of 125 μmol m^{-2} s^{-1} (LI-COR 190 SB quantum sensor, LI-COR Co.).

Adventitious and axillary shoots were established from isolated cotyledons and embryo axes, respectively. Open-pollinated seeds of Jonathan, Delicious, and Golden Delicious apple cultivars were extracted from mature fruits, surface-sterilized in a 1% sodium hypochlorite solution containing 0.1% Triton X-100 for 20 min, and rinsed three times with sterilized distilled water. The two seed coats were removed aseptically, and the embryo axis was excised. The two cotyledons and the embryo axis from each seed were cultured in three separate culture tubes. The cotyledons were cultured onto an adventitious shoot proliferation (ASP) medium consisting of half-strength Murashige and Skoog (MS) salts (2.15 g/L) (7), Staba vitamins (10 ml/L) (11), ascorbic acid (50 mg/L), casein hydrolysate (400 mg/L), BA (2 mg/L), sucrose (30 g/L), coconut milk (40 ml/L), and solidified with Difco-Bacto agar (7.0 g/L). pH was adjusted to 5.7 with 0.1 N KOH before autoclaving. Embryo axes were cultured on SPM for axillary shoot proliferation.

Fig. 1. Jonathan shoot in a culture jar showing pycnial lesions after in vitro inoculation with *Gymnosporangium juniperi-virginianae* basidiospores.

Preparation of inoculum. Cedar-apple rust galls were collected from eastern red cedar trees when telial horns were about 15 mm long and almost jellylike. The galls were stored in a freezer at −14 C.

When inoculum was needed, galls were taken from the freezer and allowed to thaw at room temperature (23 C), then soaked for 30 min in a beaker containing distilled water to induce telial horn elongation. Teliospore germination was promoted by placing the galls in a 250-ml beaker containing 50 ml of distilled water that was sealed with Parafilm to maintain high humidity. The beaker was kept at room temperature (23 C) for 15 hr to allow development of promycelia and discharge of basidiospores. A suspension of inoculum was prepared by washing galls with distilled water and collecting the basidiospores in a beaker. The concentration of basidiospores in the inoculum was adjusted to 3×10^5 spores per milliliter after counts by a hemacytometer.

Inoculation of shoots. To establish a rust inoculation system in vitro, Jonathan shoots (derived from shoot tips) were transferred either to jars (50 × 70 mm) containing fresh SPM or water agar (7 g/L Difco-Bacto agar, pH adjusted to 5.7 with 0.1 N KOH, and autoclaved for 15 min at 115 C), petri dishes (100 × 15 mm) containing water agar, or into commercial peat plugs (Castle and Cooke Techniculture, Salinas, CA). A No. 15 DeVilbiss adjustable-tip atomizer was used to inoculate shoots with a fresh spore suspension. Both jars and petri dishes were then sealed with Parafilm; the peat plug units were placed in a clear plastic bag and sealed. All were placed in the culture room under the conditions described. After 20 days, explants were examined for pycnial lesions on leaves.

To investigate the reliability of the rust inoculation system in vitro, shoots from pretested rust-susceptible (Jonathan) and resistant (Prairiefire) clones were harvested from SPM and transferred to jars containing water agar. Shoots were inoculated as described, and 20 days later, they were examined for rust symptoms.

To investigate the possible existence of somaclonal variation for rust resistance, adventitious and axillary shoots derived from cotyledons and embryo axes, respectively, of individual open-pollinated apple seeds from Jonathan, Golden Delicious, and Delicious were inoculated as described. After 20 days, shoots were examined for rust symptoms and the number and diameter (mm) of pycnial lesions on all leaves of each shoot were recorded. Then the highest number of pycnial lesions recorded on a leaf/shoot (N) and the largest diameter of pycnial lesions found on any leaf/shoot (D) were selected. The infection rating (IR) was then calculated as $IR = ND^2$, being proportional to the infected area (mm^2) of the leaves (1). The parameters N, D, and IR were subjected to analysis of variance; mean comparisons were made by Duncan's multiple range test.

RESULTS

Severe bacterial contamination occurred when shoots grown on the SPM were inoculated with the rust basidiospore suspension. Within 4–5 days of inoculation, shoots turned brown and died. Shoots grown on water agar and inoculated with the basidiospore suspension showed very little contamination. Within 10 days of inoculation, very small orange-yellowish spots (0.5–1.0 mm in diameter) developed on leaves. These spots enlarged slightly within 5–10 days and showed typical pycnial lesion development (Fig. 1). About 83% of Jonathan shoots developed disease symptoms (Table 1). Symptoms developed equally well on shoots grown in petri dishes containing water agar and those grown in peat plugs (Table 1). In general, most of the shoots that did not develop rust symptoms were those contaminated by bacteria.

The reliability of our inoculation system compared well with inoculations of pretested Jonathan (rust-susceptible) and Prairiefire (rust-resistant) shoots grown on water agar media. In these, 90% of Jonathan shoots developed pycnia within 15 days of inoculation,

Table 1. Comparison among four inoculation methods/media of Jonathan shoots with a *Gymnosporangium juniperi-virginianae* basidiospore suspension[a]

Culture methods/media[b]	No. of shoots inoculated	No. of shoots with symptoms[c]	No. of shoots without symptoms[d]	No. of contaminated shoots
Peat plugs	24	20	2	2
Culture jar/water agar	24	21	0	3
Culture jar/SPM	24	0	0	24
Petri dish/water agar	24	21	0	0

[a] Concentration was 3×10^5 basidiospores per milliliter.
[b] SPM = shoot proliferation medium.
[c] Pycnial lesions appeared within 10–14 days of inoculation.
[d] Contamination and subsequent death of shoots occurred within 4–5 days of inoculation.

whereas no rust symptoms were observed on Prairiefire shoots (Table 2, Fig. 2). Although most Jonathan shoots developed only pycnial lesions, about 10% of the shoots also developed aecial lesions after 5–6 wk in culture. Similar results were obtained from inoculated shoots of Jonathan and Prairiefire grown in peat plugs.

Adventitious shoots derived from each of three pairs of cotyledons of individual Delicious apple seeds were inoculated with rust spores and evaluated for *N*, *D*, and *IR*. No significant differences were observed for any of the three parameters between adventitious shoots derived from each of the cotyledon pairs (data not shown). When adventitious shoots derived from a single cotyledon were compared with axillary shoots derived from the embryo axis of the same seed of a Delicious, Golden Delicious, or Jonathan apple, again no significant differences were observed for any of the three parameters (data not shown). However, the *N* and *IR* values of cotyledon-derived shoots among the three cultivars were significantly different (Table 3). Cotyledon-derived Jonathan shoots had a significantly higher *N* and a significantly higher *IR* than did Golden Delicious and Delicious cotyledon-derived shoots.

DISCUSSION

The contamination of shoot-tip cultures on SPM was expected because the medium contained an abundance of nutrients for bacterial and fungal spores that grew on it very rapidly and killed shoots indiscriminately. Using water agar as a medium for shoot growth during inoculation and screening for cedar-apple rust almost eliminated contamination, and it allowed shoots to survive up to 5–6 wk, after which these cultures had to be transferred to nutrient media. This simple and effective system for in vitro inoculation of cedar-apple rust was proven to be very reliable by concurrent inoculation of shoots of known susceptible Jonathan and resistant Prairiefire clones. In these clones, pycnial lesions appeared only on Jonathan leaves and were absent on Prairiefire leaves, thereby confirming the reliability of the system.

Using the quantitative parameters *N*, *D*, and *IR*, Chen and Korban (1) evaluated greenhouse-inoculated Delicious, Golden Delicious, and Jonathan trees for cedar-apple rust resistance. The results indicated that Jonathan was more susceptible to cedar-apple rust than either Golden Delicious or Delicious cultivars. Although our cotyledon-derived shoots were obtained from open-pollinated seeds, present data also indicated that Jonathan cotyledonary shoots were more susceptible than those of Delicious and Golden Delicious.

Whether shoots were derived from the embryo axis or the cotyledonary tissue of an apple seed, variability in the amount of cedar-apple rust infection was observed. It has been reported that some degree of genetic change might occur in vitro (5), and several reports have presented evidence for induced variability in tissue culture (5,9,10). In our work with cotyledon- or embryo axis-derived shoots, we are dealing with a heterogenous tissue (the seed) as opposed to shoot-tip-derived shoots. Therefore, the observed variability in the earlier tissues should have been expected. Of course, inherent genetic differences among seeds of Delicious, Jonathan, and Golden Delicious were also present, and these should be taken into consideration.

Although inoculation of shoots in peat plugs is easy and reliable, water agar media in culture jars offer a cheaper alternative approach. Moreover, peat plug units take up more space on a culture shelf than culture tubes, jars, or petri dishes.

Further studies need to be conducted to investigate whether somaclonal variants can be identified with this cedar-apple rust inoculation system in vitro.

Table 2. In vitro inoculation of shoots from pretested rust-susceptible (Jonathan) and resistant (Prairiefire) clones with *Gymnosporangium juniperi-virginianae* basidiospores

Cultivars	No. of shoots inoculated	No. of shoots with symptoms[a]	No. of shoots without symptoms	No. of contaminated shoots
Jonathan	50	45	2	3
Prairiefire	30	0	29	1

[a] Pycnial lesions appeared within 10–14 days of inoculation.

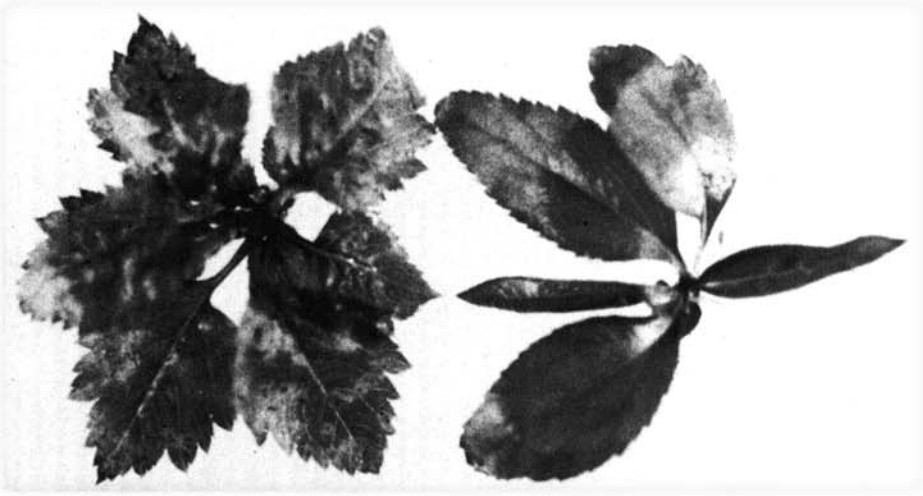

Fig. 2. (Left) Jonathan and (right) Prairiefire shoot cultures in a petri dish after in vitro inoculation with *Gymnosporangium juniperi-virginianae* basidiospores. Note the presence of pycnial lesions on Jonathan and their absence on Prairiefire.

Table 3. Lesion number (*N*), lesion diameter (*D*) and infection rating (*IR*) in different groups of adventitious shoots derived from apple cotyledons and inoculated with *Gymnosporangium juniperi-virginianae* basidiospores in vitro[x]

Seed code no.[y]	No. of shoots inoculated	Mean[z] *N*	Mean[z] *D* (mm)	Mean[z] *IR* (mm²)
D-1	15	5.2 a	1.87 a	17.90 a
D-4	15	4.2 a	1.80 a	13.95 a
D-6	15	4.6 a	1.94 a	17.53 a
D-14	15	5.1 a	1.90 a	17.93 a
G-7	15	4.9 a	1.66 a	17.73 a
J-5	15	7.1 b	1.90 a	25.27 b

[x] N = number of pycnial lesions on the most infected leaf of a single shoot, D = diameter of the largest pycnial lesion on any infected leaf of a single shoot, and IR = infection rating expressed as the area of the infected leaf and calculated as $IR = ND^2$.

[y] D-1, D-4, D-6, and D-14 are Delicious seeds, G-7 is a Golden Delicious seed, and J-5 is a Jonathan seed.

[z] Means followed by different letters are significantly different at $P = 0.05$.

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