

Grant Progress Report

North American Strawberry Growers Association

for Production Research

DEVELOPMENT OF A STANDARDIZED TECHNIQUE FOR INOCULATING
STRAWBERRY (*FRAGARIA X ANANASSA*) WITH THE ANTHRACNOSE FUNGI
COLLETOTRICHUM ACUTATUM AND *C. FRAGARIAE*

Covering the Period from

January 1, 2004 to December 15, 2004

Support in the Amount of \$1,500

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Date Submitted: December 15, 2004

NASGA ANNUAL REPORT

Project Title: **DEVELOPMENT OF A STANDARDIZED TECHNIQUE FOR INOCULATING STRAWBERRY (*FRAGARIA X ANANASSA*) WITH THE ANTHRACNOSE FUNGI *COLLETOTRICHUM ACUTATUM* AND *C. FRAGARIAE***

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Executive Summary:

The identification of disease resistant strawberry cultivars and the testing of the effectiveness of commercial and natural-based fungicides are necessary steps to reduce losses in strawberry crops. In this project our main focus was to identify standardized and optimal conditions for germination *in planta* of different phytopathogenic species and isolates (*Botrytis cinerea*, *Colletotrichum acutatum*, *C. fragariae*, *C. gloeosporioides*, *Fusarium oxysporum*, *F. oxysporum* isolate basilicum, *Phomopsis obscurans*, and *P. viticola*). The goal of our research was to provide a protocol that can be used within and between laboratories for screening strawberry cultivars for disease resistance and for testing fungicides for efficacy. For that purpose, during the *in vitro* phase of our studies we standardized several relevant aspects of the screening protocol including (1) growing fungi on Roswell Park Memorial Institute medium 1640 at $24 \pm 2^\circ\text{C}$ under cool-white fluorescent lights ($55 \pm 5 \mu\text{mol}^{-2} \text{sec}^{-1}$) as a nutrient source with a 12 h photoperiod; (2) harvesting spores at 10–14 days and washing them three times in sterile distilled water to remove innate germination inhibitors thereby significantly reducing the interval between inoculation and spore germination; and (3) adjusting the spore concentration to between 1.5×10^5 and 1×10^6 conidia/ml since higher concentrations inhibit germination. During the *in planta* phase of our studies, we developed a leaf-clearing, fungus staining technique in order to document germination conditions such as consistent application of reproducible inoculum levels and to assess rates of spore germination on the host (using detached strawberry leaves). For this purpose, small pieces of fungal-inoculated leaf were placed overnight in a solution of 1:2 absolute ethanol:glacial acetic acid to clear the leaf tissue, then the fungal tissue was stained with 0.05% acid fuchsin for two minutes, and rinsed with water. The greenhouse phase of our studies allowed us to apply our techniques to the testing the efficacy of commercial and natural-based fungicides.

DEVELOPMENT OF A STANDARDIZED TECHNIQUE FOR INOCULATING STRAWBERRY (*FRAGARIA X ANANASSA*) WITH THE ANTHRACNOSE FUNGI *COLLETOTRICHUM ACUTATUM* AND *C. FRAGARIAE*

This year we expanded the number of plant pathogenic fungi included in our studies, developed a reproducible leaf clearing technique and applied our techniques to a greenhouse fungicide study to show how the technique might have general application in screening potential fungicides for strawberry diseases.

Our three major objectives were a continuation of last year's proposal:

1. Identify parameters of plant physiology and gross morphology for consistent application of fungal spores.
2. Identify parameters of fungal physiology and early development, i.e., spore germination for consistent inoculation of test plants.
3. Identify parameters for consistent application of reproducible inoculum levels and rates spore germination on the host.

This proposal fell under two of NASGA's research priorities: (I) Cultivar Development and Testing and (II) Pest Management Strategies.

MATERIALS AND METHODS

Source of strawberry plants. Chandler strawberry plants were purchased from a commercial nursery, established in 10 x 10 cm plastic pots in a 1:1 (v/v) mixture of JiffyMix (JPA, West Chicago, IL) and pasteurized sand, and grown in a warm greenhouse for a minimum of six weeks before inoculation.

Fungal isolates. *Colletotrichum spp.* isolates from strawberry (*Fragaria ananassa* Duchesne) maintained by B.J. Smith and *Botrytis cinerea*, *Fusarium oxysporum*, *F. oxysporum* isolate basilicum, *Phomopsis viticola* and *P. obscurans* isolates maintained by D.E. Wedge (ARS, University, Mississippi) were used in these studies. Fungi were grown on potato dextrose agar (PDA, Difco, Detroit, MI) in 9-cm petri dishes and incubated in a growth chamber at $24 \pm 2^\circ\text{C}$ and under cool-white fluorescent lights ($55 \pm 5 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$) with a 12 h photoperiod.

Fungal inoculum. Conidia were harvested from 7–14 day-old cultures by flooding petri dishes with 10 ml of sterile distilled water and dislodging conidia by softly brushing the colonies with an L-shaped plastic rod. Aqueous conidial suspensions were filtered through sterile cheesecloth to remove mycelia. Conidial suspensions were adjusted with sterile water to a concentration of 1.5×10^6 conidia/ml.

Germination assays. A 24-well cell culture cluster (Corning Inc., Corning, NY) was used to establish the baseline germination times of *B. cinerea*, *F. oxysporum*, *F. oxysporum*

isolate *basilicum*, *P. viticola*, and *P. obscurans*. A sterilized 13 mm diameter microscope cover glass (Fisherbrand[®], Pittsburgh, PA) was placed at the bottom of each well and 400 μ l of Roswell Park Memorial Institute medium 1640 (RPMI, 16.2 g/l, Life Technologies, Grand Island, NY) was poured into each well. Finally, 400 μ l of the spore solution was poured into each well.

Fungi were incubated at 100% RH under the temperature and light conditions mentioned in the fungal isolates section until they reached \sim 90% germination as determined by direct observation of the wells through an IX 70 inverted system microscope (Olympus America, Inc., Melville, NY). The cover glass with the fungus attached was removed from the well and sealed to a microscope slide with transparent fingernail polish in a drop of 1% lactophenol cotton blue (stain and fixative) for subsequent observation and photography. Photographs were taken with a PM-35 DX Olympus camera (Olympus America, Inc., Melville, NY).

Detached leaf assays for fungicide evaluation. Strawberry plants (cv. Chandler) maintained in a greenhouse at previously mentioned conditions were used for this assay. No more than four hours before fungicide treatment and/or inoculation, whole leaves (with petioles) were removed from the plants. Only the second or third youngest leaves on a plant without visible signs of injury or symptoms of disease were collected. As leaves were collected they were placed in a closed container at 100% RH and carried to the laboratory. Leaves were gently washed in tap water so as not to cause injury and then placed into sterile dH₂O in 25 x 150 mm tissue culture tubes (Pyrex[®], Sigma, St. Louis, MO). All three leaflets on a leaf were inoculated by misting to the point of runoff with a conidial suspension (1.5×10^6 conidia/ml) of *C. fragariae* isolate CF-75 on the adaxial surface with a handheld pump sprayer. The experimental fungicides tested included sampangine and CAY-1 and were prepared at concentrations of 625, 1250, and 2500 ppm. These experimental fungicides were evaluated in quadruplicate with a commercial fungicide (azoxystrobin), non-inoculated controls treated with the experimental and commercial fungicides at each concentration, and an inoculated non-treated control.

Two sets of experiments were conducted: (1) a pre-inoculation treatment and (2) a post-inoculation treatment. In the pre-inoculation treatment, leaflets were inoculated with the conidial suspension, placed a dew chamber (Percival Scientific, Model I-60DL, Boone, Iowa) in the dark for 24 h at 30°C, sprayed with the experimental fungicides, and returned to the dew chamber for an additional 24 hour. In the post-inoculation treatment, leaflets were sprayed with the experimental fungicides, placed in closed containers at 100% RH and 28°C for 24 hours, inoculated with the conidial suspension, and placed in the dew chamber for 48 hours. Leaflets in both set of experiments were incubated in the dew chamber for a total of 48 hours after inoculation, then were placed in closed containers at 100% RH, 28°C, and continuous fluorescent light (Sylvania Super Saver Lite White, 34 W, 16.7 μ mol \cdot m⁻²·sec⁻¹; OSRAM Sylvania, Danvers, MA) for an additional two days before assessing for disease development. Leaf surfaces were examined microscopically after being cleared so the fungi could be selectively stained. Clearing was accomplished by placing leaf pieces in a 1:2 absolute ethanol:glacial acetic acid solution overnight (or longer; we found no deterioration in material that was stored in the clearing agent for over a month). Selective staining of fungi on the leaf surface was accomplished by submersing the leaf pieces in 0.05% aqueous acid fuchsin for 2 min, rinsing in distilled water, and mounting in a drop of water on a glass slide.

Photographs of the leaf pieces were taken with a DXC–151A digital video color camera (Hitachi Instruments, Inc., Houston, TX) attached to a BH–2 Olympus light microscope (Olympus Corporation, Marietta, GA) and captured with Bioquant 98® image-analysis software package (R&M Biometrics, Inc., Nashville, TN) for posterior macroscopic disease assessment (quantitative) and microscopic evaluation for fungal morphology (qualitative).

RESULTS

Germination assays. The standardized procedure that we previously developed improved the reliability of *in vitro* fungicide testing of three species of *Colletotrichum*. This year we expanded the procedure to include four more species in three genera (*Botrytis cinerea*, *Fusarium oxysporum*, *Fusarium oxysporum* (isolate basilicum), *Phomopsis obscurans* and *Phomopsis viticola*). As had happened last year with *Colletotrichum* spp., germination times of all species were reduced significantly by washing the spores (Table 1).

Table 1. Germination times of different fungal spp. on a polystyrene surface.

Fungus	0 washes	3 washes
<i>Botrytis cinerea</i>	18 hrs	15 hrs
<i>Fusarium oxysporum</i>	17 hrs	13 hrs
<i>Fusarium oxysporum</i> (isolate basilicum)	16 hrs	13 hrs
<i>Phomopsis obscurans</i>	96 hrs	70 hrs
<i>Phomopsis viticola</i>	96 hrs	75 hrs

Detached leaf assays for fungicide evaluation. The leaf clearing technique that we began developing last year is reproducible and easy to apply. We report here a preliminary application of the technique to a study of commercial and natural product-based fungicides. In preliminary *in planta* trials, we were able to clear strawberry leaves, successfully facilitating identification of various germination stages prior to the initiation of infection. Our leaf clearing technique shows how viable spores, germ tubes, and appressoria picked up the stain on a cleared epidermal layer (Fig. 1). Spores that did not take up the stain were assumed to be non-viable.

We restricted our study to a qualitative assessment of fungal spore germination, since our purpose was to evaluate the technique. The efficacy of the fungicide is a separate issue that we are addressing outside of this grant. Fungal spore germination response to each fungicide treatment was similar. The number of appressoria produced in the pre-inoculation treatment decreased with increasing concentrations of each fungicide (Table 1) as was easily assessed with our leaf clearing/fungal staining technique. The response to azoxystrobin is documented in Fig. 2A-C, to CAY-1 in Fig. 3A, and to sampangine in Fig. 4A. Fungal spore germination was virtually inhibited in the post-inoculation treatment at all concentrations (Table 2; Fig. 2D). We

found spore germination, but little appressorial formation, following treatment with CAY-1 at 625 ppm in the lowest concentration of the post-inoculation treatment (Fig. 3B). The higher concentrations inhibited germination (Table 2). Minimal spore germination and appressorial formation were observed when sampangine was applied at 625 ppm in the post-inoculation treatment. Spore germination, and therefore, appressorial formation were reduced significantly (Table 2; Fig 4B).

Table 2. Strawberry detached leaf assays for fungicide evaluation, pre-treatment.

Fungicide	Concentration (ppm)	Comments*
Azoxystrobin	625	Many appressoria
	1250	Some appressoria
	2500	Few appressoria
CAY-1	625	Many appressoria
	1250	Some appressoria
	2500	Few appressoria
Sampangine	625	Many appressoria
	1250	Some appressoria
	2500	Few appressoria

*All observed appressoria were collapsed.

Table 3. Strawberry detached leaf assays for fungicide evaluation, post-treatment.

Fungicide	Concentration (ppm)	Comments
Azoxystrobin	625	Very few germinated conidia; very few normal appressoria
	1250	Very few germinated conidia; very few normal appressoria
	2500	Very few germinated conidia; very few normal appressoria
CAY-1	625	Very few germinated conidia; very few collapsed appressoria
	1250	Very few germinated conidia; very few collapsed appressoria
	2500	Very few germinated conidia; very few collapsed appressoria
Sampangine	625	Very few germinated conidia; very few collapsed appressoria
	1250	Very few germinated conidia; very few collapsed appressoria
	2500	Very few germinated conidia; very few collapsed appressoria

DISCUSSION

We successfully improved the reliability of *in vitro* testing of *Botrytis cinerea*, *Colletotrichum acutatum*, *C. fragariae*, *C. gloeosporioides*, *Fusarium oxysporum*, *F. oxysporum* (isolate basilicum), *Phomopsis obscurans*, and *P. viticola*. The standardized protocol that we established after identifying important variables leading to germination *in vitro* of different fungal species ensures the establishment of optimal germination conditions of different fungal species that is reproducible within and between different laboratories for screening strawberry germplasm for disease resistant and for testing fungicide efficacy.

A persistent problem for *in planta* disease screening assays is the uncertainty of whether the resistance of the plant or the efficacy of the fungicide is causally related to “positive” results or to the failure of the fungus to perform on cue. Confirmation of *in vitro* sensitivity differences by *in vivo* sensitivity tests is essential to avoid these kinds of misinterpretations, and the information provided by *in vivo* assays will reduce that level of uncertainty and will have broad application in plant pathology. Furthermore, the fact that resistance to several fungicide classes has occurred in *Colletotrichum* spp. (Smith, 1993a, b; Peres et al., 2002, 2004) and in *Botrytis cinerea* (Wedge et al. 2001) makes an assessment of the level of the sensitivity of new fungicides

necessary. To accomplish this, methods that allow the baseline sensitivity for these fungicides are needed. However, the *in vitro* methods that have been used to evaluate the protective fungicide effects (spore germination, radial growth measurements in the presence of different fungicide concentrations and determination of the minimal inhibitory fungicide concentration) by inhibiting spore germination are inappropriate when the mechanism of the fungicide to be tested is related to infection or growth within the plant (Kunz et al., 1998).

The host plant is not required to measure the inhibition of germination. These sensitivity tests can be performed on microscope slides, agar plates, or cell culture clusters (Petsikos-Panayotarou et al., 2003; Peres et al., 2004; Wedge and Kuhajek, 1998; Wedge et al., 2001). However, a disadvantage of this method is that *in vitro* the fungicide inhibitory concentrations are smaller than those used in the field. On the other hand, even though sensitivity assays on whole plants or detached leaves are labor intensive, *in vivo* assays are particularly suited to analyze fungal sensitivity against protective and curative fungicides since fungal virulence and susceptibility of the plant tissue contribute to the results (Kunz et al., 1998).

In vitro assays may not reflect the situation under field conditions and sensitive isolates could be inaccurately classified as resistant in *in vitro* assays where infection structures are not differentiated (Birchmore et al., 1996). *Colletotrichum* species exhibit a targeted secretion of cutinolytic enzymes only during differentiation of infection structures (Podila et al., 1991). However, some fungi do not differentiate infection structures during growth on agar plates and therefore, secretion of infection related enzymes is not required. As a consequence, *in vivo* tests involving the host plant constitute an absolute necessity to corroborate sensitivity assays of fungal populations to new fungicides.

In conclusion, we have made a contribution toward the development of a protocol where standardized parameters can be established for both the strawberry plants and the plant pathogenic fungi used in *in vitro* assays and in *in planta* testing. The technique allows determination of levels of germination, appressorial development, and fungal stasis on susceptible and resistant parts of the strawberry plant or the efficacy of a fungicide applied to the plant.

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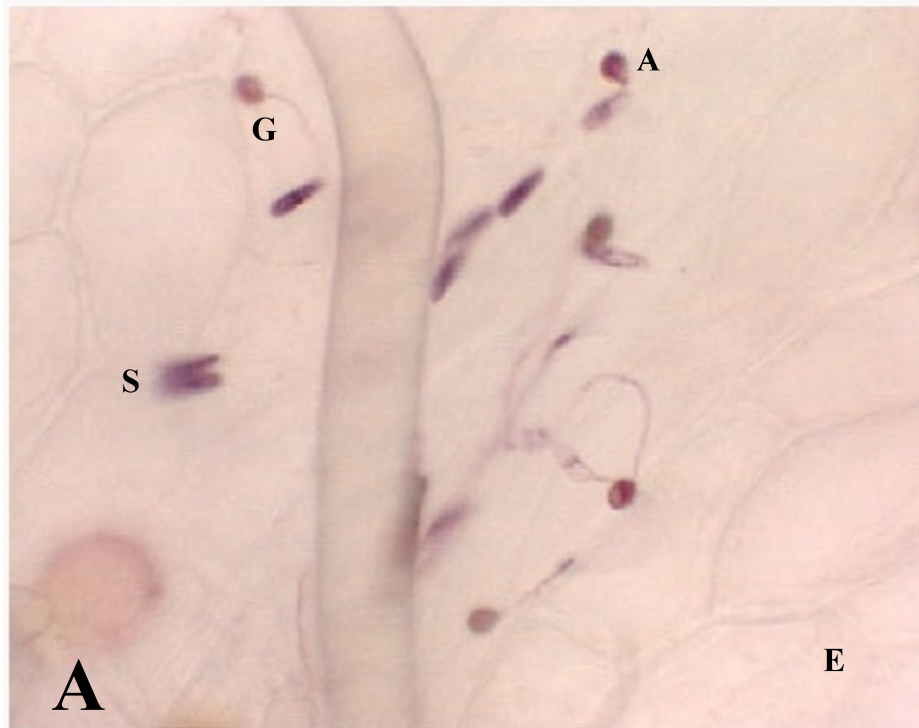


Figure 1. *Colletotrichum acutatum* on cleared leaf section of 'Chandler' cultivar of strawberry. Fungi are stained with 0.05% aqueous acid fuchsin. Various germination stages including ungerminated spores, germ tubes, hyphae, and appressoria are shown. A = appressorium, S = spores, G = germ tube, E = epidermal layer

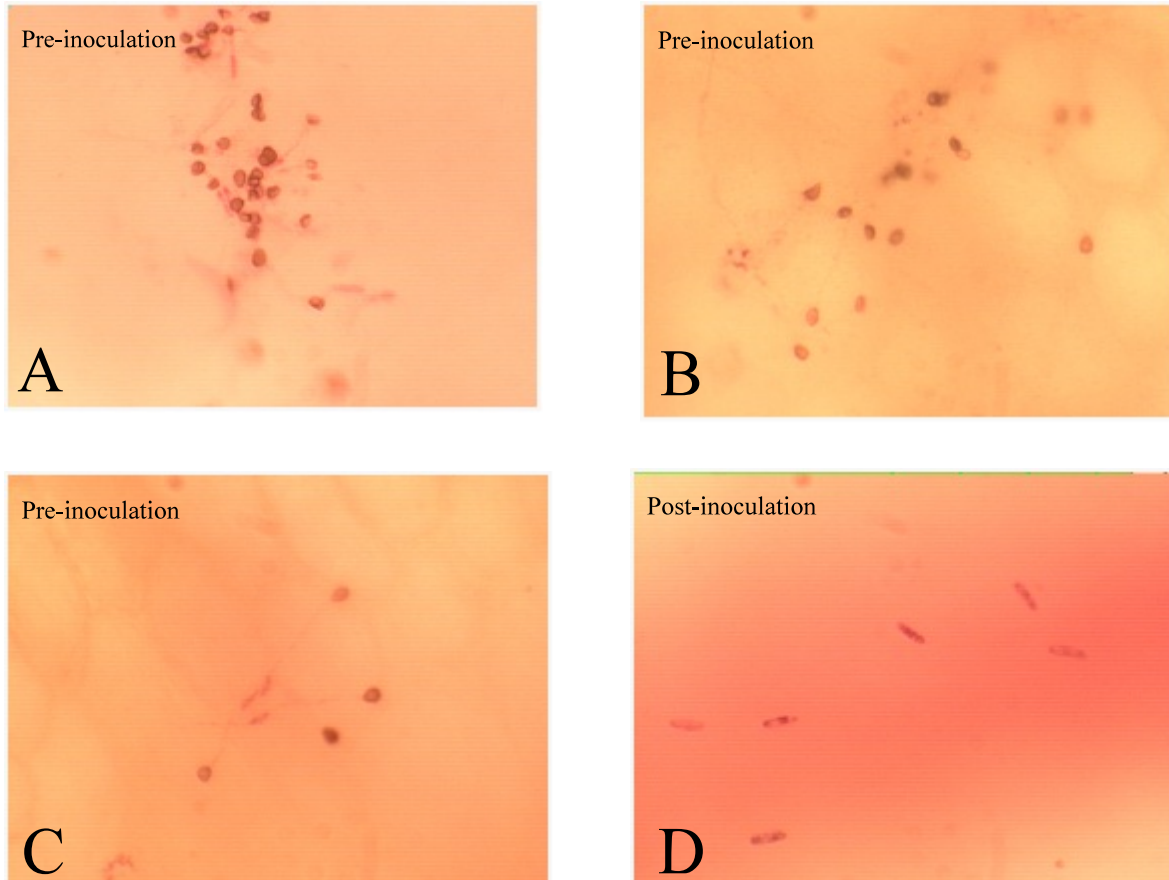


Figure 2. Cleared leaf section of ‘Chandler’ cultivar of strawberry inoculated with *Colletotrichum fragariae* isolate CF-75 and treated with azoxystrobin. Fungi are stained with 0.05% aqueous acid fuchsin. **A.** Pre-inoculation treatment sprayed with 625 ppm of azoxystrobin. **B.** Pre-inoculation treatment sprayed with 1250 ppm of azoxystrobin. **C.** Pre-inoculation treatment sprayed with 2500 ppm of azoxystrobin. **D.** Post-inoculation treatment sprayed with 2500 ppm of azoxystrobin.

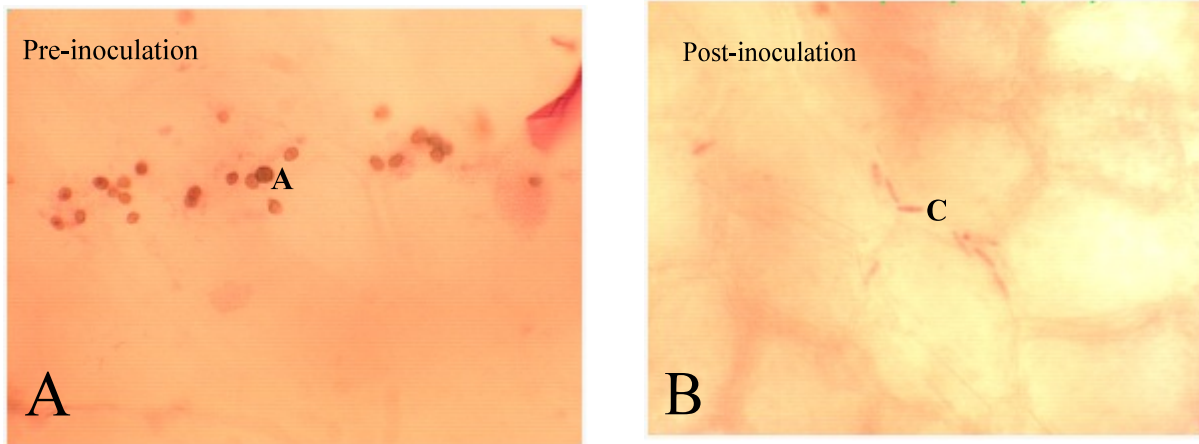


Figure 3. Cleared leaf section of ‘Chandler’ cultivar of strawberry inoculated with *Colletotrichum fragariae* isolate CF-75 and treated with CAY-1. Fungi are stained with 0.05% aqueous acid fuchsin. **A.** Pre-inoculation treatment sprayed with 625 ppm of CAY-1. **B.** Post-inoculation treatment sprayed with 2500 ppm of CAY-1. A = appressorium. C = conidium

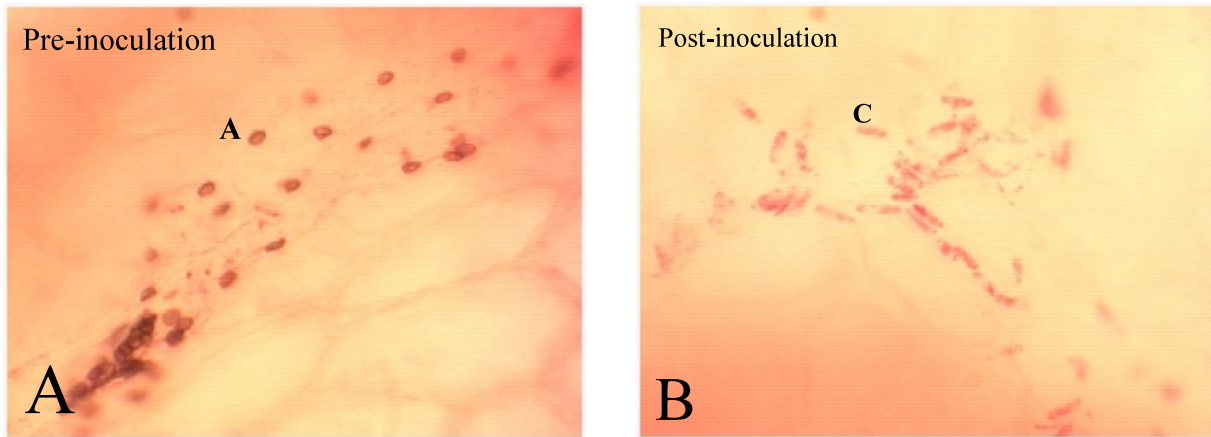


Figure 4. Cleared leaf section of ‘Chandler’ cultivar of strawberry inoculated with *Colletotrichum fragariae* isolate CF-75 and treated with sampangine. Fungi are stained with 0.05% aqueous acid fuchsin. **A.** Pre-inoculation treatment sprayed with 625 ppm of sampangine. **B.** Post-inoculation treatment sprayed with 2500 ppm of sampangine. A = appressorium, C = conidium