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OSMIUM VAPOR PRETREATMENT OF GNOMONIA INFECTED LEAVES

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Abstract

A study was conducted to determine if pretreatment with osmium tetroxide (OsO_4) vapor prior to the conventional preparation procedure would increase the retention of fungal structures on leaf surfaces as observed with scanning electron microscopy (SEM). Leaves of black walnut (Juglans nigra L.) were inoculated with conidia of Gnomonia leptostyla (Fr.) Ces. and de Not., the etiological agent that causes anthracnose of walnut. Following lesion development, leaves were either conventionally prepared with immersion in fixative, ethanol and critical-point dried or vapor-fixed with OsO_4 before conventional specimen preparation. Data indicate that significantly more fungal structures were present on OsO_4 vapor-fixed leaf samples than on conventionally prepared samples.

KEY WORDS: Fungi, plant disease, leaf surface ecology, Marssonina juglandis, Julgans nigra, Gnomonia leptostyla.

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Introduction

During the last few years a great variety of preparation techniques have been reported for the observation of leaf surfaces (1,4,5) with scanning electron microscopy (SEM). Since the primary use of SEM is to examine surface structure, the main purpose in specimen preparation is to insure that the leaf surface and epiphytic fungi remain in their lifelike, original positions (5). Before examining the specimen, preparatory procedures are carried out to preserve the delicate fungi in their natural shape with minimum alterations (1). Conventional preparation procedures employing aqueous glutaraldehyde, osmium tetroxide (OsO_4) and ethanol can rearrange the position of fungi on leaf surfaces as well as destroy plant epicuticular waxes, creating artifacts (7). More spatially authentic results could be obtained if fungi on leaf surfaces were examined in a fresh, hydrated condition (6). Scharf (12) has described procedures involving observations of plant material in uncoated and unfixed states with success. In our experience, the low accelerating voltages that he used can limit resolution in many samples while fungal particles can yield "charging" and severely affect the quality of micrographs obtained. Cryotechniques employing cryostages have been used successfully to avoid specimen contact with aqueous solutions (11). Prohibitive cost and availability usually preclude their use. Osmium vapor pretreatment has been effectively used to preserve lipid cell components (6) and was used for immobilization of certain diffusible substances (3). A study was designed to determine if treatment with OsO_4 vapor prior to conventional preparation would increase the retention of fungal structures on leaf surfaces.

Materials and Methods

Fungal Culture

The walnut anthracnose fungus Gnomonia leptostyla (Fr.) Ces. and de Not. (Marssonina juglandis (Lib.) Magn.) was grown in petri plates on oatmeal agar (20 g of instant oatmeal, 5 g of dextrose, 20 g of agar and 1 L of distilled water) for 2-3 wk at 21°C under a 12-h photoperiod (2). Conidia were washed from the plates, collected by centrifugation, and the concentration was adjusted to 10^6 conidia per milliliter.

Plant Material

Seedlings of Juglans nigra L. were grown in environmental growth chambers (EGC). The light intensity was 26,900 lux and the photoperiod 16 h.

The temperature was maintained at 24° ± 1°C day and 21° ± 1°C night. Relative humidity was 70 ± 10%.

A conidial suspension was atomized onto mature, fully expanded leaves that were then covered with plastic bags for 36 h at 20°C. Following the removal of the bags, seedlings were maintained at EGC conditions described above.

Electron Microscopy

Following inoculation, incubation and lesion expression, inoculated and uninoculated leaflets were prepared with the following conventional methodology: fixed in 3% glutaraldehyde for 18 h in a 0.1 M phosphate buffer at pH 7.2, post-fixed in 1% OsO_4 (in a pH 7.2 phosphate buffer) for 2 h, washed in phosphate buffer, dehydrated in 35, 50, 85, 95, 99% and absolute ethanol (3X) for 5 min each and critical-point-dried (CPD) with liquid CO_2 in a Tousimis Autosamdri Model 810 critical-point-drier (8,10).

Equal numbers of samples were prepared with the following procedure: leaf samples were harvested and suspended over 2% OsO_4 solution within a closed container 15x100mm(9). Osmium vapor pretreatment lasted for 1-1/2 h. Pretreated samples were fixed in glutaraldehyde, dehydrated in ethanol, and CPD as described above. As an additional control, fresh leaf tissue was sampled. All specimens were mounted on stubs and sputter-coated with gold. Only upper surfaces were examined with a Hitachi S-500 SEM set at 20 kV and 5mm working distance with a 40-degree tilt. Twenty samples from each preparation procedure were analyzed for numbers of conidia present per unit area scanned by the electron beam at a magnification of ∿ 1000X (approximately 2500 μm^2).

Conidial counts were made of 20 specimens per preparation procedure with 2 samples per specimen. Data were analyzed by using analysis of variance and means were separated by the least significant difference (LSD) test, $P < 0.05$ (13).

Results and Discussion

Lesions formed on walnut leaves 36 h following inoculation. SEM examination of conventionally prepared specimens revealed conidia and germ tube (gt) growth (Fig. 1). Germ tubes appeared collapsed (Fig. 2). The leaf surfaces, while stable under the electron beam, appeared grainy. Conidial counts of specimens are shown in Table 1. Counts indicated that 10 times more conidia were present on osmium pretreated specimens than on conventionally prepared leaf surfaces. Osmium pretreated specimens displayed conidia and germ tubes that were turgid and appeared normal in shape and size (Figs. 3 and 4). Leaf surfaces were stable under the beam and appeared to be smoother than conventionally prepared leaf samples.

SEM examination revealed conidia with germ tubes on fresh, hydrated leaf samples (Fig. 5). Germinating conidia were apparent but very ephemeral while leaf surfaces were "charged" and extremely vulnerable to beam damage. Since walnut leaves had profuse guttation and were extremely hydrated, we feel these factors contributed to the instability of the fresh, epidermal cells at magnifications over X1000. Means of conidial counts from fresh, hydrated specimens were not significantly different than the means of specimens pretreated with OsO_4 vapor (Table 1). Uninoculated leaves did not display any conidia of G. leptostyla.

Table 1. Retention of Gnomonia leptostyla conidia on Juglans nigra upper leaf surfaces following various specimen preparation procedures.

Preparation Procedures	Conidial Count* Number/2500 μm^2
Conventional procedure (Aqueous glutaraldehyde, OsO_4 ethanol and critical point)	1a**
OsO_4 vapor pretreatment + conventional procedure	10b
Fresh, hydrated	11b

*Mean of 20 specimens with 2 sample areas/specimen.
**Values followed by the same letter are not significantly different ($P < 0.05$ LSD).

We can only speculate as to the chemical mechanism that seems to be acting to fix, in place, G. leptostyla conidia to black walnut leaf surfaces. Perhaps osmium binds the lipids of the epicuticular leaf wax to the fungal cell wall. More detailed research is required to determine the mechanism.

Since cryopreparation techniques were not performed in this study, comparisons cannot be made with the OsO_4 vapor pretreatment described above. However, due to the simplicity and relative low expense, of this procedure, the authors believe the osmium vapor pretreatment could be a reasonable alternative to cryopreparation techniques.

This study does indicate the need for special techniques to observe leaf fungi with SEM in their authentic in vivo positions while maintaining adequate control over artifacts induced by conventional fixation procedures and electron beam/specimen interactions.

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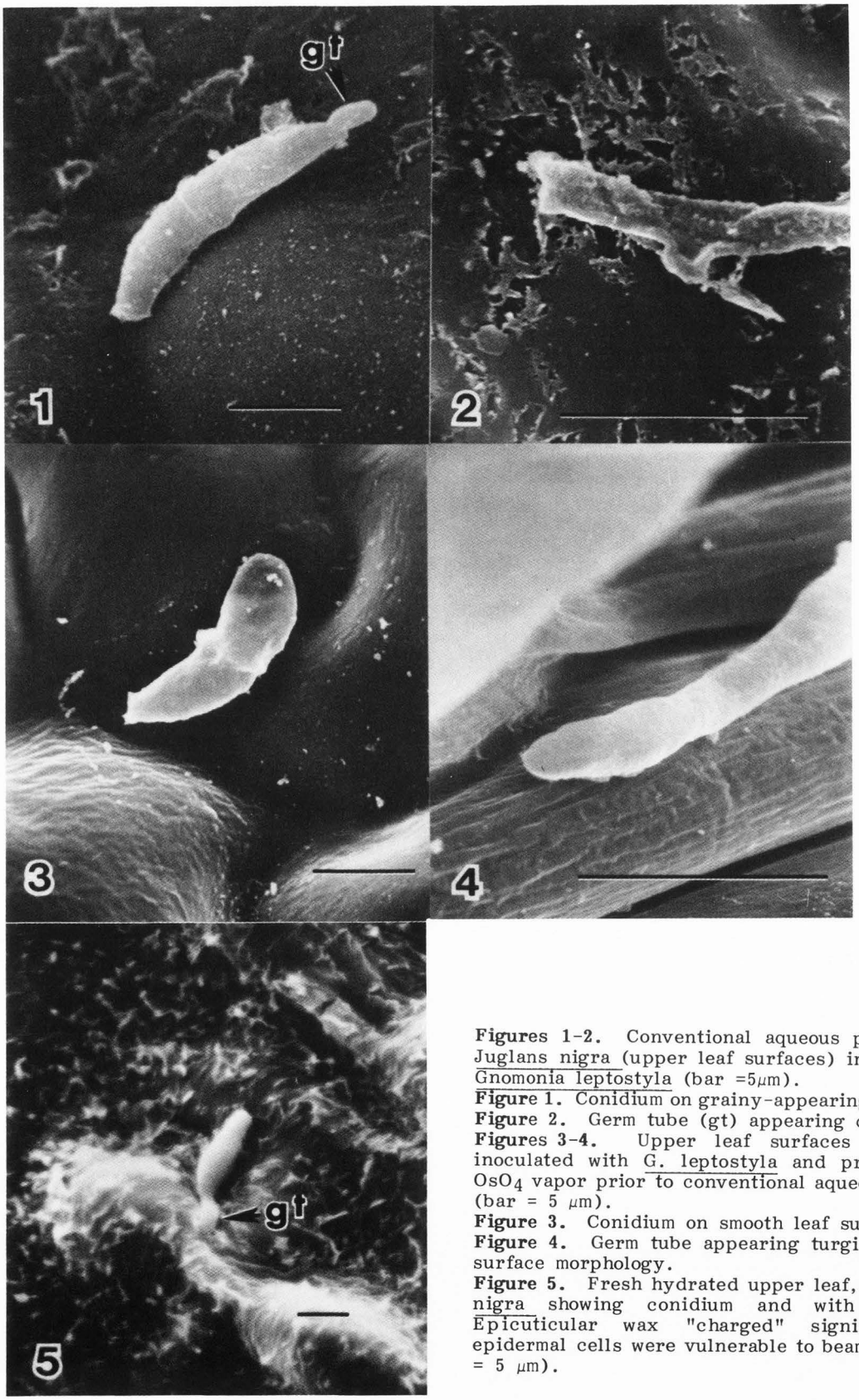

Figures 1-2. Conventional aqueous preparation of Juglans nigra (upper leaf surfaces) inoculated with Gnomonia leptostyla (bar =5μm).
Figure 1. Conidium on grainy-appearing leaf surface.
Figure 2. Germ tube (gt) appearing collapsed.
Figures 3-4. Upper leaf surfaces of J. nigra inoculated with G. leptostyla and pretreated with OsO_4 vapor prior to conventional aqueous procedure (bar = 5 μm).
Figure 3. Conidium on smooth leaf surface.
Figure 4. Germ tube appearing turgid with normal surface morphology.
Figure 5. Fresh hydrated upper leaf, surface of J. nigra showing conidium and with germ tube. Epicuticular wax "charged" significantly and epidermal cells were vulnerable to beam damage (bar = 5 μm).

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Discussion with Reviewers

Reviewer I: Was tilt correction used in the SEM measurements?
Authors: Yes, we used the Hitachi tilt correction module.

Reviewer I: Why wasn't freeze drying used as an alternative?
Authors: We have never attained acceptable results with freeze dried fungal specimens. Detail is always lost with numerous artifacts.

Reviewer III: Was fresh leaf tissue sputter coated with gold?
Authors: Yes, all samples from all three treatments were sputter coated with approximately the same thickness of gold.

Reviewer I: Why do you refer to the OsO_4 vapor technique as cheap?
Authors: While the price of osmium has recently increased, we were comparing the relative expense of osmium and its use with conventional SEM ambient temperature stages to the substantial, initial investment required to purchase cryogenic stages.

Reviewer I: Why aren't low magnification micrographs presented to show differences?
Authors: Low magnification micrographs are not available since we were observing many samples and were working under the limitation of time.

Reviewer II: Could you be more specific in the differences in the appearances of the leaf surface between the osmium pretreated samples and those conventionally prepared?
Authors: Leaf surface wax (epicuticular) of samples prepared conventionally seemed to be damaged as noted in Fig. 2, while wax on samples prepared with OsO_4 vapor pretreatment were smooth (Fig. 3). Perhaps OsO_4 vapor pretreatment served to preserve epicuticular wax.

Reviewer II: Why doesn't Fig. 5 show more conidia present on the fresh hydrated leaf?
Authors: While several conidia were present, fresh hydrated conidia "charged" prohibiting an acceptable micrograph.

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