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# Infection of *Nicotiana* species by the anthracnose fungus, *Colletotrichum orbiculare*

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**Abstract**

*Colletotrichum gloeosporioides* f. sp. *malvae*, isolate Biomal®, ATCC 20767, was originally isolated from round-leaved mallow (*Malva pusilla*) and developed as a weed biocontrol agent. Ribosomal DNA sequence analysis was recently used to re-classify this fungus as *C. orbiculare*, which is an aggregate species with a number of formae speciales. Several morphological features of ATCC 20767 were examined that were consistent with those described for *C. orbiculare*, and inoculation of a number of *Nicotiana* species and several cultivars of *N. tabacum* showed that this fungus was pathogenic to many of these previously undescribed hosts. Spore germination and appressorium formation were higher on tobacco than previously observed on round-leaved mallow. The pathogen produced melanized appressoria on *N. tabacum* leaves that formed preferentially at the anticlinal epidermal cell wall. A symptomless phase of infection persisted for 72–96 h postinoculation, during which time the fungus first produced a spherical infection vesicle from an infection peg, and then large primary hyphae which grew through the epidermal cells. The large primary hyphae were highly constricted at the points of penetration of the host cell walls. Thin secondary hyphae appeared at 96–120 h postinoculation coinciding with the appearance of light green, water-soaked spots and the formation of acervuli. The infection of tobacco by *C. orbiculare* ATCC 20767 is not a non-specific interaction but appears to follow an intracellular hemibiotrophic infection process that is very similar to that established for the *C. orbiculare* infection of round-leaved mallow, cucurbits and beans.

## Introduction

*Colletotrichum gloeosporioides* f. sp. *malvae* (Penz.) Penz. & Sacc. is a plant-pathogenic fungus that has shown potential as a biological control agent of round-leaved mallow (*Malva pusilla*) and related weeds in the Malvaceae (Mortensen, 1988). This organism was registered as the first mycoherbicide in Canada under the tradename BioMal® (Makowski and Mortensen, 1992). In addition to studies on the infection of target weeds (Makowski, 1993; Morin et al., 1996; Wei et al., 1997), several studies were conducted on non-target plants to ensure that unintended plant damage did not occur if the fungus was applied commercially (Makowski and Mortensen, 1998; Mortensen and Makowski, 1995, 1997). Of ten non-target crops tested, only safflower showed susceptibility with significant damage occurring with only one cultivar, and therefore BioMal® was considered safe for most crops but was not recommended for use with safflower (Mortensen and Makowski, 1997).

The taxonomic status of *Colletotrichum* species has been largely based on pathogenicity and certain morphological features (Sutton, 1980, 1992). However, morphological characteristics may be variable in culture, and there is an overlap of phenotypes, which has made these criteria not always reliable. DNA sequence comparisons of the internal transcribed spacer regions (ITS) and other portions of the ribosomal DNA (rDNA) have been used to examine a number of *Colletotrichum*

species (Bailey et al., 1996; Sherriff et al., 1994; Sreenivasaprasad et al., 1996). An examination of the conidial morphology and the ITS and D2 sequences of the rDNA of isolates of *Colletotrichum* from plants in the Malvaceae showed that all the isolates of *C. gloeosporioides* f. sp. *malvae* from *M. pusilla*, including the BioMal® isolate, should be regarded as *C. orbiculare* (Berk. & Mont.) von Arx (syn. *C. lagenarium* (Pass.) Ell. & Halst.) (Bailey et al., 1996). Bailey et al. (1996) noted that *C. orbiculare* is pathogenic to a number of important crops, such as beans and cucurbits, and suggested that this identification should elicit a re-examination of the specificity of BioMal®.

In the course of studying tobacco anthracnose, we incidentally discovered that the BioMal® isolate (ATCC 20767) of *C. gloeosporioides* f. sp. *malvae*, hereafter referred to as *C. orbiculare* 20767, could also infect certain *Nicotiana tabacum* cultivars. As plants in the genus *Nicotiana* have not been described previously as natural hosts for *C. orbiculare*, an examination of certain characteristics of the fungus, its interaction with different *Nicotiana* species and the infection process in *N. tabacum* and *N. benthamiana* were undertaken.

*Table 1*. Reaction of *Nicotiana* species to *Colletotrichum orbiculare* 20767

| Plant | Seedling reaction | Mature plant reaction |
|---|---|---|
| *N. alata* PI 42334 | S | PRL |
| *N. attenuata* PI 555476 | S | S |
| *N. benthamiana* WAU | S | S |
| *N. glutinosa* PI 555507 | GT | R |
| *N. gossei* PI 230953 | S | R |
| *N. pauciflora* PI 555546 | S | S |
| *N. plumbaginifolia* PI 302478 | R | R |
| *N. rustica* PI 555554 | S | PRL |
| *N. sylvestris* PI 555570 | PR | PRL |
| *N. tabacum* cv. Burley21 | S | R |
| *N. tabacum* cv. Delgold | R | — |
| *N. tabacum* cv. Petit Havana | S | — |
| *N. tabacum* cv. Samsun | R | R |
| *N. tabacum* cv. Xanthi | S | R |

S: production of 40 to several hundred small water-soaked lesions per leaf that dried to a papery appearance. PRL: partial resistance with lower leaves damaged with 40 to several hundred lesions but 0–3 lesions per leaf on upper leaves. PR: partial resistance with 2–3 lesions per leaf on inoculated plants. GT: rot of growing tip but no leaf lesions. R: no symptoms. —: not determined. Control plants were inoculated with water and did not show any disease symptoms. Results from at least three separate inoculations.

## Materials and methods

### *Fungal material*

*Colletotrichum orbiculare* 20767 was cultured on potato dextrose agar (PDA, Difco, Detroit, MI) or sodium chloride–yeast extract–sucrose agar (SYAS) (Manandhar et al., 1986) under continuous fluorescent lighting at 25 °C. Conidia for inoculation of plants were obtained from 7-day-old cultures grown on SYAS. The size of conidia was determined by measuring the length and width of 50 spores harvested from PDA cultures after 7 days' growth.

### *Inoculations*

Plants of *Nicotiana* species (Table 1) were grown from seed in Pro-Mix (Premier Horticulture Inc., Red Hill, PA) at 25 °C with a 16 h photoperiod at an intensity of 200 μmol $s^{-1}$ $m^{-2}$ until the sixth leaf stage for seedlings or at the beginning of the flowering stage for mature plants. Entire plants were inoculated by spraying until runoff with a suspension of $2 \times 10^6$ conidia $ml^{-1}$ in sterile distilled water. The plants were immediately sealed in plastic bags for 24–30 h, incubated at 25 °C in the growth chamber for up to 9 days, and observed each day for disease symptoms. Each inoculation was repeated at least three times.

### *Microscopy*

Sections of the two youngest mature leaves of sixth leaf stage plants containing infection sites were excised and stained with 0.05% (w/v) trypan blue in lactophenol and heated over a flame for approx. 10 s until boiling. Stained leaf pieces were examined by light microscopy and photographed (Nikon Labophot). Randomly selected portions of the stained tissues of *N. tabacum* cv. Xanthi and *N. benthamiana* were observed to determine the percentage of conidial germination, percentage of germ tubes producing appressoria, location of appressoria, and the infection process. The size of appressoria was determined by measuring the length and width of 50 appressoria that had developed on inoculated leaves of *N. tabacum* cv. Xanthi. Conidial germination and appressorial formation results were based on at least 50 observations on each of three or more inoculated plants. Proportions calculated from these observations were analysed in Student's *t*-tests for differences between host species

after angular transformation, which was calculated as the arcsine of the square root of the proportion.

## Results

Inoculation of five varieties of *N. tabacum* and nine other species of *Nicotiana* with *C. orbiculare* 20767 showed that three of the varieties of *N. tabacum* and all the other *Nicotiana* spp., except *N. plumbaginifolia*, were susceptible as seedlings (Table 1). However, several of these plants were either resistant or partially resistant when inoculated at maturity (i.e., early flowering stage). In the resistant reaction with seedlings or mature plants, conidia germinated and produced appressoria, but no further disease development occurred, and there were no visible hypersensitive reactions or obvious papillae. In the susceptible reaction, anthracnose symptoms appeared as small water-soaked spots that enlarged to 2–4 mm diameter (Figure 1) and then dried to give a thin papery appearance. The timing of symptom appearance for most plants was similar to that of *N. tabacum* cv. Xanthi leaves, which showed water-soaked lesions at day 5 after inoculation followed by the appearance of acervuli at day 7. In *N. benthamiana*, however, the same symptoms appeared at day 4 and acervuli appeared at day 6 after inoculation. *N. tabacum* cv. Petit Havana had identical symptoms that occurred in only 3 days and acervuli appeared 5 days after inoculation. The most severely infected species were *N. attenuata* and *N. pauciflora*, which developed over 300 small lesions (approx. 1–2 mm diameter) per leaf that quickly coalesced resulting in complete death of leaves, petioles and stems by day 5 for both seedlings and mature plants. *Colletotrichum orbiculare* 20767 was readily isolated from surface-sterilized, infected leaves of the susceptible *Nicotiana* species, and the re-isolated fungus appeared identical to that of the culture first used for inoculation. Conidia from the re-isolated fungus were also able to infect tobacco seedlings.

*Figure 1*. Typical symptoms due to infection of tobacco by *C. orbiculare* 20767. The appearance of small (2–4 mm), water-soaked spots on a leaf of *N. benthamiana* 4 days after inoculation.

An examination of the conidia of *C. orbiculare* 20767 revealed that they were straight to slightly curved with obtuse ends (Figures 2A,B) and measured 13.1 ± 1.6 μm (mean ± standard deviation) in length and 5.4±0.7 μm in width. The conidia germinated, and appressoria formed by 12 h after inoculation (Table 2). No septation of the conidium was observed after germination. The appressoria were occasionally clavate (Figure 2A), but the vast majority were more ovate and often slightly lobed (Figure 2B). The average length and width of appressoria that developed on leaves was 7.3 ± 1.3 μm and 5.2 ± 0.9 μm, respectively. There appeared to be a preference for appressoria forming at the epidermal cross-walls (i.e., the anticlinal epidermal walls), which occurred in 78 ± 5% of germlings on *N. benthamiana* and 76 ± 2% of germlings on *N. tabacum* cv. Xanthi. Figures 2A and B also show some of the variation in length of the germ tubes, which may be related to preferential penetration at the anticlinal cell wall.

A higher percentage of spores germinated within 12 h on *N. tabacum* cv. Xanthi than on *N. benthamiana*, but there was no significant difference in germination on these two species by 24 h (Table 2). Although a higher percentage of the germinated spores produced

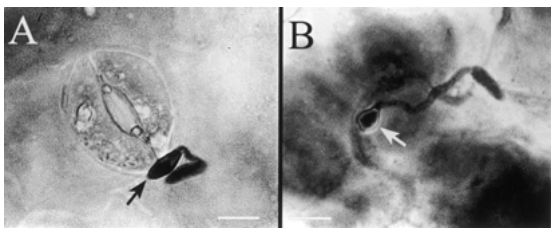


*Figure 2*. Germination of conidia of *C. orbiculare* 20767 on (A) *N. tabacum* cv. Xanthi (B) *N. benthamiana* leaf surfaces at 24 h postinoculation. Arrow indicates the appressorium. Bar = 10 μm.

appressoria on *N. tabacum* cv. Xanthi rather than on *N. benthamiana*, a lower percentage of the appressoria appeared to be successful in causing infection, as indicated by the lower number of lesions developing on *N. tabacum* cv. Xanthi (Table 2).

The infection process and appearance of fungal structures in the host tissues were similar in *N. tabacum* and *N. benthamiana*. Growth in the host epidermal cell began with a globose infection vesicle that was produced between 24 and 48 h postinoculation, typically near the edge of an epidermal cell (Figure 3A), and from this, primary hyphae emerged and a septum formed (Figure 3B). The primary hyphae grew and began to branch in the infected epidermal cell (Figure 3C). Upon reaching the plant cell wall, the primary hyphae became slightly bulbous, and showed

*Table 2*. Conidial germination, appressorial formation and disease severity of *Colletotrichum orbiculare* 20767 inoculated on leaves of *Nicotiana benthamiana* and *Nicotiana tabacum* cv. Xanthi

| | 12 h | | 24 h | | 120 h |
|---|---|---|---|---|---|
| | Germination[a] | Appressoria[b] | Germination | Appressoria | Lesions/cm$^2$[c] |
| *N. benthamiana* | 59% | 42% | 72% | 72% | 20.0 |
| *N. tabacum* cv. Xanthi | 76% | 63% | 78% | 93% | 5.9 |
| *t*-test[d] | $P < 0.01$ | $P < 0.05$ | $P = 0.37$ | $P < 0.001$ | $P < 0.001$ |

[a]Mean percentage based on three plants with at least 50 conidia on inoculated leaf surfaces of each plant.
[b]Mean percentage based on three plants with at least 50 germinated spores on inoculated leaf surfaces of each plant.
[c]Number of lesions per square centimeter of inoculated leaves.
[d]*P* values of less than 0.05 in Student's *t*-tests indicate significant differences between the two *Nicotiana* species. Angular transformation (arcsine of the square root of the percentage/100) was applied to germination and appressorial percentages prior to analysis.

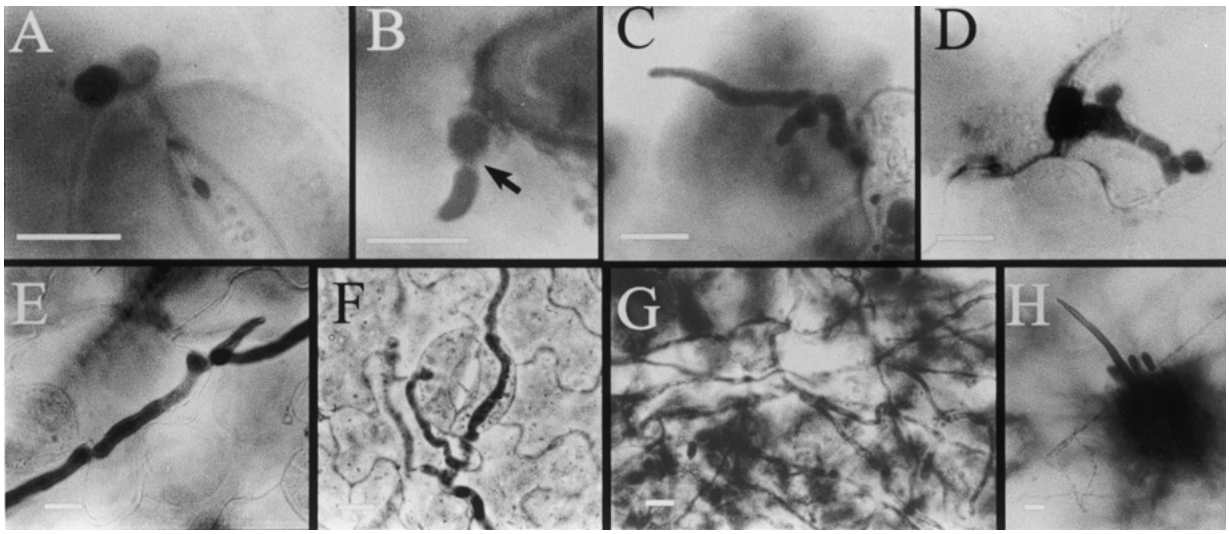


*Figure 3*. Infection of *N. tabacum* and *N. benthamiana* by *C. orbiculare* 20767 (A) Early development of rounded infection vesicle at 48 h postinoculation on *N. tabacum* cv. Xanthi. (B) Emergence of large primary hyphae from an infection vesicle at 48 h postinoculation on *N. tabacum* cv. Xanthi. Arrow denotes septum. (C) Large primary hyphae growing in initially infected epidermal cell at 48 h postinoculation on *N. tabacum* cv. Xanthi. (D) Large primary hyphae in infected epidermal cell at 72 h postinoculation on *N. tabacum* cv. Xanthi. (E) Large primary hyphae growing through several adjacent epidermal cells showing hyphal constrictions at the host cell walls at 72 h postinoculation on *N. benthamiana*. (F) Invasion of stomatal guard cells by large primary hyphae with constrictions at the host cell walls at 72 h postinoculation on *N. tabacum* cv. Xanthi. (G) Intercellular growth of secondary hyphae in necrotic leaf tissue at 120 h postinoculation on *N. tabacum* cv. Xanthi. (H) An acervulus with a single seta on the surface of leaf tissue at 120 h postinoculation on *N. tabacum* cv. Xanthi. Bar = 10 μm.

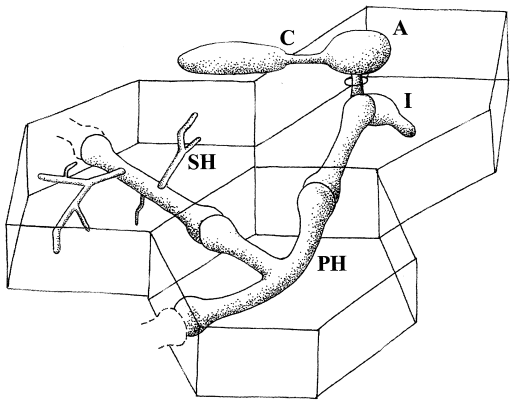


*Figure 4.* Diagrammatic view of infection of tobacco epidermal cells by *C. orbiculare* 20767. Shown are a conidium (C) with a germ tube leading to an appressorium (A), a penetration peg entering an epidermal cell below the appressorium, an infection vesicle (I) in the epidermal cell, large primary hyphae (PH) growing from the infection vesicle and penetrating adjacent epidermal cells with hyphal constrictions at the site of penetration, and thin secondary hyphae (SH) emerging from the primary hyphae.

constrictions where they penetrated through the wall (Figures 3D,E). No visible disruption to the host cell appeared at this time. Although most primary hyphae grew through the non-photosynthetic epidermal cells, they also showed the same pattern of growth when penetrating the photosynthetic guard cells of the stomata (Figure 3F). Thin secondary hyphae, arising from the primary hyphae, were observed at 96–120 h postinoculation, and could be distinguished from primary hyphae by widespread intercellular growth and a lack of hyphal constrictions as they penetrated the host cell walls (Figure 3G). Growth of secondary hyphae corresponded with the appearance of water-soaked lesions that eventually dried. Masses of conidia with melanized setae formed on the leaf surface (Figure 3H). Figure 4 is a diagrammatic view of the various stages of infection prior to acervulus formation and is based on numerous observations of infected tissues.

## Discussion

When Bailey et al. (1996) proposed that *C. gloeosporioides* f. sp. *malvae* isolates from *M. pusilla* should be identified as *C. orbiculare*, only the ranges of conidial length and shape were given based on observations of 14 different isolates. The specific morphological features of the isolate 20767 were not reported. The description of *C. orbiculare* given by Sutton (1992) stated that sclerotia and setae may be abundant or absent, appressoria may be ovate, clavate to irregular, 6.5–16 × 5.5–10 μm, and conidia are straight or slightly curved, 10–15 × 4.5–6 μm. This description agrees with all the corresponding features in *C. orbiculare* 20767. Sutton (1980) differentiated *C. gloeosporioides* from *C. orbiculare* primarily based on the former having conidia usually not more than 4.5 μm wide, whereas the latter had conidia 4.5–6 μm wide. Isolate 20767 had conidia that were 5.4 ± 0.7 μm in width, and these conidia remained aseptate after germination, which is also characteristic of *C. orbiculare* and has been observed by several research groups working with this same isolate (Bailey et al., 1996; Morin et al., 1996; Wei et al., 1997).

During its development as a biocontrol agent of round-leaved mallow, *C. orbiculare* 20767 had been tested on a number of non-target crops but not tobacco (Makowski and Mortensen, 1998; Mortensen, 1988; Mortensen and Makowski, 1995; 1997). The plants that were tested in those studies were primarily chosen based on their relatedness to the weed host and the likelihood that they would be exposed if the fungus was used commercially as a weed biocontrol agent. As a result, many plant species were excluded. Because tobacco is susceptible to *C. orbiculare* 20767, the use of this fungus as a biocontrol agent in or near crops of tobacco, particularly at the seedling stage, may be inadvisable. The inoculations in this study, however, were done under laboratory conditions where circumstances are most favorable for infection and may not reflect the level of damage that would occur under field conditions. Also, a commonly planted tobacco variety, Delgold, was found to be resistant at the seedling stage, and therefore *C. orbiculare* 20767 could be applied as a weed biocontrol agent in or near tobacco fields if resistant varieties are grown.

Anthracnose of tobacco is a seedling disease that is present in all tobacco-growing areas and is caused by *C. destructivum* (syn. *C. nicotianae*, *C. tabacum*, *C. gloeosporioides*) (Cronin, 1958; Farr et al., 1989; Shew and Lucas, 1991). The symptoms on tobacco produced by *C. orbiculare* 20767 appear to be identical to those caused by *C. destructivum* (Shew and Lucas, 1991). There are no reports that *C. orbiculare* or any other *Colletotrichum* species, except *C. destructivum*, can attack tobacco in the field (Farr et al., 1989). However, it is not entirely unexpected that tobacco could be infected by other *Colletotrichum* species. Cronin (1958) inoculated *N. tabacum* and *N. rustica* with 17 different isolates of various *Colletotrichum*

species, each obtained from a different host species, and noted that *C. fragariae*, *C. nigrum* and *C. trifolii* could infect *N. tabacum*. *C. fragariae* had a wide host range, including *N. rustica*, tomato and cucumber, and was just as virulent on tobacco as the isolates of the tobacco anthracnose fungus, *C. destructivum*, obtained from tobacco. A comparison of the current work with data from our previous study of *C. orbiculare* 20767 infection of round-leaved mallow (Wei et al., 1997) shows that the percentage spore germination and number of appressoria formed per germinated conidium of *C. orbiculare* 20767 was greater on tobacco than on round-leaved mallow, which is the original host, implying that the fungus begins the infection process on tobacco seedlings as effectively as on round-leaved mallow.

Although *C. orbiculare* and *C. destructivum* produce similar symptoms in tobacco, the interaction of tobacco with *C. orbiculare* or *C. destructivum* is significantly different. In tobacco, *C. destructivum* produces irregularly shaped primary hyphae only in the initially infected cell, and then thin secondary hyphae emerge from the tips of the primary hyphae and grow into adjacent host cells (Cronin, 1958; Shen and Goodwin, 2001). In addition to a different infection process, these two fungi have differences in their host range. Inoculation of seedlings of 60 species of tobacco with *C. destructivum* showed that *N. plumbaginifolia* PI 302478 and *N. glutinosa* PI 555507 were more susceptible to anthracnose than *N. tabacum* cv. Burley 21 (Sievert, 1972). However, in our tests, seedlings of these two tobacco species were rated resistant and partially resistant, respectively, to *C. orbiculare*.

Morphological and molecular data indicate that several *Colletotrichum* species should be grouped together as *C. orbiculare* (Bailey et al., 1996; Sherriff et al., 1994; Sreenivasaprasad et al., 1996). This includes *C. lindemuthianum* from beans, *C. orbiculare* from cucurbits, *C. trifolii* from alfalfa and *C. gloeosporioides* f. sp. *malvae* from mallow, which have been proposed to be different *formae speciales* of *C. orbiculare* (Bailey et al., 1996). A common feature of all the fungi in the *C. orbiculare* aggregate species is that they have an intracellular hemibiotrophic infection strategy with an initial biotrophic phase where globose intracellular infection vesicles and primary hyphae are observed in epidermal cells (Bailey et al., 1992; 1996; O'Connell et al., 1985). This phase usually persists for 4–6 days, during which time, no symptoms are visible. These structures have been observed in infected round-leaved mallow (Wei et al., 1997), watermelon (Anderson and Walker, 1961), cucumber (Busch and Walker, 1958), cantaloupe (Dargent and Touzé, 1974), beans (Bailey et al., 1992; O'Connell et al., 1985) and now in several species of tobacco. Additional common features among these interactions are the presence of hyphal constrictions in primary hyphae as they penetrate the plant cell walls and the subsequent appearance of thin secondary hyphae without hyphal constrictions corresponding with the necrotrophic phase of infection and the appearance of disease symptoms. The fact that *C. orbiculare* has a virtually identical process of infection in tobacco as in these other plants indicates that infection of tobacco is not simply a non-specific infection of a plant, but involves fungal structures associated with all the stages of the intracellular hemibiotrophy normally observed with infections by *C. orbiculare*.

In practical terms, the commercial use of *C. orbiculare* 20767 as a weed biocontrol agent may not be adversely affected by the finding that tobacco seedlings can become infected. However, this study does show that there may be many more plants susceptible to weed biocontrol agents than is currently indicated by primarily testing only plants most likely to come in contact with the agent or plants taxonomically related to the weed.

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