Localization and movement of mineral oil in plants by fluorescence and confocal microscopy

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Abstract

Fluorescence and confocal laser scanning microscopy were explored to investigate the movement and localization of mineral oils in citrus. In a laboratory experiment, fluorescence microscopy observation indicated that when a ‘narrow’ distillation fraction of an nC23 horticultural mineral oil was applied to adaxial and opposing abaxial leaf surfaces of potted orange [Citrus × aurantium L. (Sapindales: Rutaceae)] trees, oil penetrated steadily into treated leaves and, subsequently, moved to untreated petioles of the leaves and adjacent untreated stems. In another experiment, confocal laser scanning microscopy was used to visualize the penetration into, and the subsequent cellular distribution of, an nC24 agricultural mineral oil in C. trifoliata L. seedlings. Oil droplets penetrated or diffused into plants via both stomata and the cuticle of leaves and stems, and then moved within intercellular spaces and into various cells including phloem and xylem. Oil accumulated in droplets in intercellular spaces and within cells near the cell membrane. Oil entered cells without visibly damaging membranes or causing cell death. In a field experiment with mature orange trees, droplets of an nC24 agricultural mineral oil were observed, by fluorescence microscopy, in phloem sieve elements in spring flush growth produced 4–5 months and 16–17 months after the trees were sprayed with oil. These results suggest that movement of mineral oil in plants is both apoplastic via intercellular spaces and symplastic via plasmodesmata. The putative pattern of the translocation of mineral oil in plants and its relevance to oil-induced chronic phytotoxicity are discussed.

Key words: Apoplast, confocal laser scanning microscopy, fluorescence microscopy, localization, mineral oil, penetration, phloem, symplast, translocation.

Introduction

Petroleum-derived spray oils have been used to control susceptible phytophagous pests and diseases of plants for >100 years (Agnello, 2002). Contemporary highly refined products are classed as horticultural mineral oils (HMOs) and agricultural mineral oils (AMOs) (Kuhlmann and Jacques, 2002). Both have minimum paraffin contents of 60% and minimum unsulphonated residue values of 92%. The difference between them relates to the equivalent n-paraffin carbon number (nCy: Beattie et al., 2002) range between their 10% and 90% distillation points. The range for an HMO is <=6 whereas the range for an AMO is >6 (Agnello, 2002; Kuhlmann and Jacques, 2002).

An important constraint to the use of HMOs and AMOs is their potential to be phytotoxic. Such phytotoxicity, although uncommon when products are used correctly, has been quite extensively studied and the causes are generally well understood (Ebeling, 1950; Riehl, 1981; Davidson et al., 1991; Hodgkinson et al., 2002). Naturally occurring acidic compounds in oils, and such compounds formed largely by oxidation of unsaturated molecules present in most contemporary HMOs and AMOs, cause acute effects that are generally observed as burns and necrotic lesions shortly after application of oil to plants. These effects are primarily related to cell death due to disruption of membranes by the acidic compounds, and are influenced by oil quality, dose, and ambient conditions, particularly
temperature, humidity, and ultraviolet light (Hodgkinson et al., 2002). Such symptoms are perhaps the most widely recognized form of phytotoxicity and are commonly associated with oils with relatively high levels ($\geq 8\%$) of unsaturated molecules. However, acute phytotoxicity such as rapid leaf drop may also result from the impact of high dose applications of HMOs and AMOs on plant growth regulators (Johnson et al., 2002). These dramatic effects are most common when sprays are applied to moisture-stressed plants, immediately before or after extreme ambient temperatures and humidity. The risk increases with increasing median nCy values, and is generally related to the persistence of isoalkanes and cycloalkanes on sprayed surfaces. Such molecules comprise $\geq 92\%$ of molecules in HMOs and AMOs. Under such extreme conditions, slowly volatilizing oil deposits lead to ethylene-induced abscission (Johnson et al., 2002).

Under average conditions and in the absence of moisture stress, high doses of products, particularly high molecular weight HMOs and AMOs, may cause chronic symptoms, such as loss of vigour, leaf and fruit drop, and reduced flowering and fruit set (Furness and Maelzer, 1981; Beattie et al., 1989). The causes of this type of phytotoxicity are poorly understood and the manner and consequences of the movement of petroleum-derived spray oils within plants under different conditions have been largely ignored despite their importance in selecting the appropriate oil to use. Although the penetration and movement of herbicidal oils in some plants have been demonstrated since the 1950s (van Overbeek and Blondeau, 1954; Gauvrit and Cabanne, 1993), the last detailed studies on the movement of pesticidal oils into, and within, plants were reported by Knight et al. (1929) and Rohrbaugh (1934, 1941) for citrus, and by Young (1933, 1934, 1935, 1936, 1941) for apples and vegetables. These important studies have not received the attention they deserve given the potential for oil to interfere with gas exchange and translocation of water and metabolites. Furthermore, the conclusions reached by these authors differed. Knight et al. (1929), and Young in his publications, claimed that saturated petroleum-derived oils move between cells, and into xylem and living cells (including phloem). Rohrbaugh (1934) claimed that saturated oils did not move into cells and dismissed the observations reported by Knight et al. (1929). Oddly, Rohrbaugh (1934, 1941) did not cite Young’s publications, and Ebeling (1950, p. 199), in his classic chapter on spray oils in ‘Subtropical entomology’, did not discuss these different views. These differences of opinion, which have not been resolved for 60 years, may have been related to methodology.

This paper reports on the use of fluorescence microscopy (FM) and confocal laser scanning microscopy (CLSM) to visualize the penetration, subsequent movement, and cellular distribution of mineral oils into and within citrus leaves, petals, and stems. An nC23 HMO, a distillation fraction of another nC23 HMO, and an nC24 AMO were used in this study to clarify the putative patterns of movement of mineral oils in plants and the implications of such movement with respect to chronic phytotoxicity. Due to the fact that mineral oils themselves are non-fluorescent and cannot be visualized directly by FM and CLSM, the lipophilic dye Nile red was used to label the oils or stain sections cut from oil-treated and untreated tissues to emit fluorescence under certain light excitations.

Materials and methods

Plants

Potted Valencia and mature Washington navel orange (Citrus × aurantium L. (Sapindales: Rutaceae)) trees and rootstock seedlings of Citrus trifoliata L. on the University of Western Sydney’s Hawkesbury campus at Richmond (33° 62’ S, 150° 75’ E) were used in the experiments.

Mineral oils and fluorescent dye

Three mineral oils were used. One was an nC23 HMO (Caltex Summer Spray Oil; Caltex Australia Pty Ltd) with specifications similar to the nC23 HMO used by Herron et al. (1995). Its unsulphonated residue value was $\geq 94\%$ and it contained $\leq 0.9\% v/v$ of one or more non-ionic emulsifiers. The second was an nC24 AMO (SK EnSpray 99; SK Corporation, Korea). Its unsulphonated residue value was $\geq 99.8\%$ and it contained $\leq 0.9\% v/v$ of non-ionic emulsifiers. The third was a ‘narrow’ distillation fraction from nC23 Ampol D-C-Tron NR, a product similar to Caltex Summer Spray Oil. It had a median nCy number of 21 and an nCy range of 2 between the initial and final distillation points (Liu et al., 2002). The glossary in Beattie et al. (2002) defines relevant spray oil terms used in this paper and more widely. The fluorescent dye Nile red (C20H14N2O2; 9-diethylamino-5H-benzo[a]phenoxazine-5-one) (Molecular Probes Inc., Eugene, OR, USA) was used for both FM and CLSM experiments.

Fluorescence microscopy

FM detection of mineral oil was carried out with an Olympus BX-60 microscope with exciter filter BP 460–490, dichroic mirror DM-505, barrier filter BA515IF, $\times 10$ eyepieces and $\times 40$ UPlanApo objectives (Olympus Australia Pty Ltd, North Ryde, NSW, Australia). The fluorescence of mineral oil was viewed under FM with blue excitation.

Confocal laser scanning microscopy

CLSM analyses were performed with a Leica SP2 microscope (Leica, Heidelberg, Germany) fitted with an argon ion and two helium neon lasers. The beam splitter was DD488/543 (double dichroic, reflects at 488 and 543 nm). The fluorescence of mineral oil was excited at 488 nm and collected at 510–600 nm. Specimens were photographed using a combination of emitted light and transmitted light. The resultant images were overlaid in some cases to determine the location of the fluorescence signal, and thence the localization of oils. During image acquisition, each line was scanned eight times and averaged. Images were projected from the stack of images in the z-direction using the maximum intensity mode.

Fluorescence induction of mineral oils

The lipophilic dye Nile red was used to induce fluorescence of mineral oils. Two experiments were undertaken to evaluate method specificity. Firstly, mineral oils were labelled by dissolving...
The experiment was based on mature sprayed mature orange trees. Medium- to long-term movement of samples in the presence of oil with those in the absence of oil. A 500 ml multi-purpose hand-held Selecta Spray trigger sprayer (Plaspak JWS Pty Ltd, Seven Hills, NSW, Australia) was used to spray droplets of agitated 2% v/v oil-in-water emulsion to glass slides. The slides were then examined under FM and CSLM to assess the fluorescence of Nile red-labelled oil. Ten glass slides were measured for each of the microscopes. Secondly, the sprayer was used to spray 2% v/v aqueous oil emulsions to run-off to potted Valencia orange trees or rootstock seedlings that were subsequently held in a laboratory at 22 °C. Freehand cross-sections cut from leaves and stems of sprayed trees or seedlings 1 d after spraying were stained with 100 µg ml⁻¹ Nile red solution for 1 h. Stained sections were mounted on glass slides, each covered with a coverslip, and then examined with FM and CSLM to determine the fluorescence of Nile red-stained oil in plant tissues. At least 10 leaves and stems were measured for each microscope. Negative controls were included in all experiments. The specificity was checked by comparing samples in the presence of oil with those in the absence of oil.

Medium- to long-term movement of nC24 HMO in sprayed mature orange trees

The experiment was based on mature ∞3-m-high Washington navel orange trees in an orchard located on the campus. It was part of a larger experiment examining phytotoxicity related to oil concentration in sprays, frequency of application, and time of application during late summer and autumn 2000. For this experiment, FM was used to determine the extent of nC24 HMO movement within sprayed plants. Of 15 trees sampled, five were sprayed once with 7% v/v emulsions on 14 March, five were sprayed on four occasions (8 and 22 February, and 14 and 29 March) with 1.75% v/v emulsions, and another five were from the unsprayed control. Each oil-sprayed tree was sprayed with 20 l of emulsion applied at 1000 kPa with a 120-l-capacity Hardi Wheelbarrow sprayer fitted with a spraying gun with solid stream nozzles (Hardi Pumps and Sprayers Pty Ltd, Sydney, NSW, Australia). The movement of oil within sprayed mature 1999 spring growth flushes produced 4–5 months before spray application, and unsprayed mature spring 2000 flushes produced 16 months after spray application, was determined by viewing the distribution of oil in 10 randomly chosen mature 1999 flushes and 10 similarly chosen mature 2000 flushes, on each tree from 8–13 August 2001. The youngest leaf and its petiole on the former, the oldest leaf and its petiole on the latter, and the stem located immediately below their nodes were excised. Five transverse sections were cut from each of the tissues and stained by Nile red for FM observation as described previously. Tissue was considered oil-positive if oil fluorescence was observed in any of the sections. Data were analysed using GenStat 7.1 software (Payne et al., 2003). Binomial testing was performed to compare the difference between treatments. The average minimum and maximum temperatures at Richmond from 1 January 2000 to 30 September 2001 were 9.9 °C and 23.8 °C, respectively. The minimum and maximum temperatures during this period were −5.8 °C and 45 °C, respectively (Bureau of Meteorology).

Short-term penetration and translocation of micro-applicator-applied nC24 HMO-fraction in orange leaves, petioles, and young stems

The narrow distillation fraction from Ampol D-C-Tron NR and potted Valencia orange trees were used for this experiment. The trees were the same age and dimensions as those used to observe the fluorescence of mineral oils labelled or stained by Nile red as described previously. Thirty-two uniform trees were divided randomly into eight groups, each comprising four trees. These groups of trees were used, one for each time, for FM measurements conducted 1, 4, 16, 24, 36, 48, 72, and 96 h after oil application. One flush was selected from each tree for oil treatment. A micro-applicator (Burkard Scientific, Uxbridge, UK) was used to apply 100 µl drops of oil to abaxial and adaxial surfaces near the tips of each of five mature terminal leaves on each flush. A glass rod was used to gently spread each droplet over a 1×2 cm area on each surface. For each time, all four flushes within a group were excised; a single leaf, petiole, and stem on each flush were sectioned. Sections of midveins were taken from the area 2.5–3 cm below the oil-treated area. Petioles were sectioned across their centres and sections of stems were cut immediately beneath the proximal end of the petioles. FM observations, determination of oil presence in tissue, and data analysis were the same as described in the section above.

Visualization of mineral oils labelled or stained by Nile red

Nile red-labelled mineral oil on glass slides fluoresced yellow-gold under FM with blue excitation, but negative controls of either mineral oil or Nile red alone on slides resulted in the absence of such fluorescence signal (data not shown). When freehand sections of Valencia orange leaves and stems sprayed with mineral oil were stained by Nile red solution, such fluorescence was also observable (Fig. 1a, leaf; Fig. 1b, stem), which is distinguished from the fluorescence of unsprayed sections stained by Nile red solution (Fig. 1d). CLSM observation indicated that Nile red-labelled mineral oil on slides emitted fluorescence at 510–600 nm under excitation at 488 nm (fluorescent green in this paper); no such fluorescence was produced from samples of non-Nile red-labelled oils or Nile red solution (data not shown). Fluorescence was detected from tissues that were sprayed with oil emulsion and stained by Nile red solution (Fig. 1c, leaf midvein). No such fluorescence was observed in sections of all negative controls, but the cuticle emitted weak fluorescence similar to that of the mineral oil (data not shown). These results demonstrated the specificity of Nile red for mineral oil. Consequently, Nile red was used in subsequent experiments to trace the movement and localization of mineral oil in plants.

Visualization of nC24 AMO penetration into, and localization in, citrus leaves and stems

The nC24 AMO was fluorescently labelled by dissolving Nile red in the oil at a concentration of 100 µg ml⁻¹. Ten C. trifoliata seedlings were used, with five being used for foliage treatment and five for stem treatment. Five hundred micrograms of the fluorescently labelled oil was applied with a micro-applicator to a 2 cm² (1×2 cm or 0.8×2.5 cm) area on the abaxial surface of a distal leaf, or an internodal region of a stem 15–18 cm above ground level, using the method as described in the previous section. Freehand cross-sections were cut from the oil-treated leaves and stems, and viewed under CLSM at fixed times after oil application. The penetration of oil through stomata and cuticle of oil-treated leaves and stems, and the localization of oil between and within cells of tissues were observed and imaged using a ×63 water-immersion objective.

Results

Fluorescence of mineral oils labelled or stained by Nile red

Nile red-labelled mineral oil on glass slides fluoresced yellow-gold under FM with blue excitation, but negative controls of either mineral oil or Nile red alone on slides resulted in the absence of such fluorescence signal (data not shown). When freehand sections of Valencia orange leaves and stems sprayed with mineral oil were stained by Nile red solution, such fluorescence was also observable (Fig. 1a, leaf; Fig. 1b, stem), which is distinguished from the fluorescence of unsprayed sections stained by Nile red solution (Fig. 1d). CLSM observation indicated that Nile red-labelled mineral oil on slides emitted fluorescence at 510–600 nm under excitation at 488 nm (fluorescent green in this paper); no such fluorescence was produced from samples of non-Nile red-labelled oils or Nile red solution (data not shown). Fluorescence was detected from tissues that were sprayed with oil emulsion and stained by Nile red solution (Fig. 1c, leaf midvein). No such fluorescence was observed in sections of all negative controls, but the cuticle emitted weak fluorescence similar to that of the mineral oil (data not shown). These results demonstrated the specificity of Nile red for mineral oil. Consequently, Nile red was used in subsequent experiments to trace the movement and localization of mineral oil in plants.
Medium- to long-term movement of nC23 HMO in mature orange trees

Results of FM observation of oil distribution in mature trees sprayed with either a single 7% nC23 HMO spray in March 2000 or four 1.75% sprays of the same oil during February and March 2000 are summarized in Table 1. Oil was present in leaves and stems of sprayed mature 1999 spring flush growth and unsprayed 2000 spring flush growth produced after oil sprays (Fig. 2a–c); there were significant differences in oil presence in the leaf midveins (P<0.04) and stems (P=0.005) of 1999 spring flush, and the petioles (P=0.047) and stems (P<0.001) of the spring 2000 flushes between unsprayed trees and trees sprayed once with 7% and four times with 1.75% of nC23 HMO, suggesting that oil that penetrated surfaces after spray application subsequently moved through and from sprayed flush growth to unsprayed flush growth. The amount of oil in the spring 1999 stems (photographs not presented) was visibly less than in the spring 2000 stems, but the distribution of oil in these stems was not uniform. At the time of sampling in spring 2001, oil in the mature 2000 spring flush was present in the petiole of the proximal leaf but absent in the petiole of the younger terminal leaf (Fig. 2b, d).

Short-term penetration and translocation of oil in orange leaves, petioles, and young stems

Movement of oil molecules in the nC21 distillation fraction of nC23 Ampol D-C-Tron NR from the treated leaf tips to midveins and petioles in untreated regions of the leaves, and to adjacent untreated stems occurred within 48 h of oil application (Table 2). There were significant differences in the presence of oil in the leaf midveins (P<0.001), petioles (P<0.001), and stems (P=0.041) between times post-application. Oil rapidly penetrated abaxial and adaxial surfaces of leaves, and travelled to the leaf mesophyll and midveins 2.5–3 cm below the oil-treated area of the leaves 1 h post-application and, subsequently, to the leaf petioles, and the adjacent stems within 48 h post-application. Oil droplets were visible in the mesophyll, cortex, phloem, xylem, and pith of leaves and stems (Fig. 3), suggesting
putative movements of oil via both vascular bundles and intercellular spaces in plants.

Visualization of nC24 AMO penetration into, and localization within, citrus leaves and stems

CLSM allowed visualization of penetration of mineral oil into leaves and stems, and its subsequent localization within and between plant cells. Cross-sectional images of oil-treated leaves showed that most oil penetrated into the mesophyll through stomata, and subsequently moved within intercellular spaces. During such movement, oil molecules diffused into the cytoplasm of both palisade and spongy cells (Fig. 4a). When applied to stems, oil moved through both xylem and intercellular spaces between epidermal cells; oil molecules diffused into the cytoplasm of both the epidermal and the parenchymatous cells (Fig. 4b). Figure 5a, b shows the distribution of oil droplets in a leaf midvein 8 h, and in a stem 24 h, respectively, post-application. A large accumulation of oil droplets was detected in the outer cortex of the midvein, where oil droplets were found in intercellular spaces and cells, with
some cells and intercellular spaces being filled with oil. Only a few oil droplets were scattered in cells and intercellular spaces in the inner cortex. More oil droplets were visible in intercellular spaces than in cells. Most oil droplets in cells were distributed in the protoplast near the membranes, adjacent to the oil droplets in intercellular spaces (Fig. 5a). Such localization was also observed in the stem, where oil droplets were visible in cells and intercellular spaces in cortex, phloem, and xylem. More oil droplets were detected in intercellular spaces than in cells; oil droplets in cells were mainly located near cell membranes. Oil was also present in the sieve elements of phloem (Fig. 5b).

### Discussion

In this study, the use of both FM and CLSM to trace the movement and localization of mineral oil in plants was demonstrated. Both fluorescence and confocal microscopy have increasingly been used to probe physiological process in plants (Kathryn and Oparka, 1996; Fricker and Oparka, 1999; Paddock, 1999; Wymer et al., 1999). However, studies using these techniques to investigate pesticide movement within plants appear to have been reported on few occasions (Dybing and Currier, 1961; Liu, 2004), probably due to the fact that most pesticides are non-fluorescent and cannot be easily visualized by fluorescence microscopy (Liu, 2004). In the experiments reported here,
the lipophilic dye Nile red was employed to label or stain non-fluorescent mineral oils to generate fluorescence. Nile red has been widely used recently to detect lipid compounds in bacteria and fungi (Kamisaka et al., 1999; Spiekermann et al., 1999; Weber et al., 1999; Hansen et al., 2000), to study tissue localization of certain compounds such as proteins, lipids, and phenolic compounds in plants (Kathryn and Oparka, 1996; Hutzler et al., 1998). In the present study, the fluorescence of Nile red-labelled or -stained mineral oils was easily distinguished from Nile red-stained cell membrane and cellular lipid constituents, making detection of mineral oil in plants feasible. This property may be due to the circumstances in which it was used and a change in its polarity, since it has been shown that Nile...
Movement of mineral oils from treated tissues to untreated tissues, including movement from sprayed leaves and stems produced >1 year after the application of spray emulsions, was observed. Mineral oil was also observed between cells, in xylem, and in the protoplasms of cells, including phloem. This oil did not cause visible damage to cell membranes and therefore cell death. Such damage is traditionally associated with acidic compounds in oils, and such compounds formed on exposure of unstable molecules to light after the application of spray. The results confirm the observations of Knight et al. (1929) and Young (1933, 1934, 1935, 1936, 1941) and clearly demonstrate that saturated mineral oil molecules do not move into living cells (Rohrbaugh, 1934, 1941) were incorrect.

The present results demonstrated that mineral oil moves within intercellular spaces or penetrates into cells after entering citrus leaves, petioles, and young stem tissue. However, oil droplets accumulate in intercellular spaces and within cells during movement, resulting in more oil droplets being distributed in the outer mesophyll of the leaves and in the cortex of the stems than in the inner mesophyll, vascular bundle, and pith of the tissues. This is possibly due to the lipophilic nature of mineral oil molecules. Despite it being widely known that foliar uptake of lipophilic pesticides is much faster than that of hydrophilic pesticides (de Ruiter et al., 1993), the high affinity of lipophilic compounds for lipoidal cuticles and membranes prevents their desorption from the cuticle and cells to the internal tissues (de Ruiter et al., 1993; Liu, 2004). Other properties of mineral oil such as molecular weight and type, and the influences of environmental factors such as temperature and plant morphology, may also affect movement of oil molecules.

The movement of pesticides in plants has historically been characterized as either apoplastic, by which they move in intercellular spaces, and within and outside cell walls, and in xylem and non-living fibres, or symplastic, by which the movement occurs through plasma membranes, and within protoplasm through the plasmodesmata of living cells. Based on the present observations and those of Knight et al. (1929) and Young (1933, 1934, 1935, 1936, 1941) it is evident that movement of AMOs and HMOs in plants is apoplastic and symplastic. However, movement is predominantly apoplastic while symplastic movement is slower, with the oils being relatively immobile compared with most other types of agrichemicals, many of which penetrate cell membranes quite easily and subsequently move rapidly within protoplasm (Malcolm, 1989). After penetrating into leaves and stems, oil mainly travels in intercellular spaces. During this period, penetration into plasma membranes, which is the first stage of symplastic movement, is slow compared with apoplastic movement. With time, oil can enter, accumulate, and remain in phloem for long periods, the duration of which is governed by ambient temperatures, oil properties, dose, and the nature of plant tissues.

The present observations of the movement of mineral oils within plant tissues provide an insight into oil-induced phytotoxicity. They suggest that excessive accumulation and persistence of HMO and AMO molecules within plant tissue cause this form of phytotoxicity by blocking translocation and distribution of metabolites and nutrients. This is possibly the major contributing factor to chronic phytotoxicity associated with contemporary HMOs and AMOs. Therefore, it is beneficial to investigate the factors affecting the movement and persistence of mineral oils in plants to minimize the risk of oil-induced phytotoxicity. Such phytotoxicity has been historically linked to dose and increasing distillation temperatures (Ebeling, 1950; Riehl, 1981) now expressed as median nCy values (Beattie et al., 2002).

References


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