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Cuticular Retention *in Vitro* and Localization of Zn after a Foliar Application of Zinc-containing Fungicides[1]

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Abstract **The cuticular retention of zinc contained in 4 fungicides (Antracol, Calyram, M-Special, and Polyram Combi) and zinc chloride was studied using isolated pear leaf cuticles (*Pyrus communis* L. cv. Passe Crassane).The retention of Zn by the cuticular discs was significant after a 24-hour immersion in each fungicide (0.2% w/v). The distribution coefficient, calculated as the ratio of the zinc retained by the cuticles (in μg/g) to the zinc content of the immersion liquid (in μg/ml), varied from 25.1 to 88.2 for the fungicides, but was only 5.7 in the case of $ZnCl_2$. Laser probe mass spectrography indicated that Zn supplied with the fungicides remained superficially localized 24 hours after a foliar application on maize (*Zea mays* L. cv. Dekalb 202); Zn was detectable only in the surface zone corresponding to a maximum depth of 30 μm from the adaxial leaf surface.**

Many Zn-containing fungicides are currently sprayed on leaves for plant protection from pathogens. Some observations suggest that the Zn in these fungicides remains localized at the leaf surface level (6) but further work is needed to specify its exact localization. This study was undertaken with 2 objectives: a) to determine cuticular retention of the Zn provided by the fungicides by using isolated pear leaf cuticles; and b) to determine the localization of Zn in maize leaves treated with Zn-containing fungicides by laser probe mass spectrography.

Materials and Methods

Chemicals. The commercial and common names of the Zn-containing fungicides are listed in Table 1.

Determination of the cuticular retention. Cuticular discs (10-mm diameter) from the upper surface of fully expanded 'Passe Crassane' pear leaves, harvested in August in an orchard near Grenoble, France, were enzymatically isolated from the underlying tissues using 2% pectinase and 0.2% cellulase buffered at pH 3.8 as previously described (3). Samples of 21 dry cuticular discs were immersed for 24 hr in 100 ml 0.2% (w/v) of one of the different fungicides or $ZnCl_2$; each solution contained Tween 20 at 0.1% (v/v) as a surfactant. The pH values were 5.5, 4.9, 8.9, 6.5, and 4.4 with Antracol, Calyram, M-Special, Polyram Combi, and $ZnCl_2$, respectively. The period of immersion was long enough to be sure that equilibrium was attained, as was previously shown with Zn as $ZnCl_2$ (4). The experiments were carried out in a gyrotory water bath shaker (New Brunswick Model G 76- speed 5.25) at 25° C. At the end of the immersion period, the cuticles were washed 5 min in 100 ml of deionised water by continous agitation. The cuticular discs were recovered in a nylon net and dried on filter paper. Each batch of 21 discs was divided into 3 samples of 7 discs each, which were separately mineralized and analysed for Zn by atomic absorption spectrometry. Thus, 3 determinations were obtained for each chemical (fungicide or zinc chloride). The results were subjected to a one-way analysis of variance.

Localization studies. The localization experiments were carried out on 15-day-old plants of 'Dekalb 202' maize hydroponically grown in a climate chamber (1). Three or five 2-μl droplets

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Table 1. Names[z] and Zn content of the fungicides used in both experiments.

Fungicide Name	Formulation
Calyram	mixture of **captan** (22%) and **metiram** (38%); 63.8g Zn/kg powder
M-Special	mixture of **captan** (24%) and **zineb** (36%); 82.8g Zn/kg powder
Polyram Combi	**metiram** (80%); 144g Zn/kg powder
Antracol	**propineb** (70%); 157.5g Zn/kg powder
Captan	N-(trichloromethylthio)-3a, 4, 7, 7a- tetrahydrophtalimide
Zineb	Zn ethylene-1, 2- bisdithiocarbamate
Metiram	complex of **zineb** and polyethylene thiuram disulphide, containing 80% zineb
Propineb	polymer of Zn propylenebisdithiocarbamate

[z]Names in italics are commercial names; names in boldface are common names.

were deposited in the middle of the lamina of the 3rd or the 4th leaf, aligned in a direction parallel to the main vein. Temperature was 25°C and relative humidity about 60%. The treated leaf was sectioned 24 hr after the fungicide application and washed twice for a total time of 2 min in 1 liter of deionised water. Immediately after washing, small segments (about 3 × 10 mm) were cut from the area which received the droplets. The samples were immersed in freon 22 cooled with liquid nitrogen, freeze-dried, and stored in a desiccator until measurement.

Laser probe mass spectrography. The principle of the laser probe mass spectrography has been recently described (2). The recent use of an original Nd/YAG laser head has improved the laser ionization efficiency and decreased the detection limit to about 10^{-13}g (5). The operating conditions were as follows: the light pulse consisted of UV radiation (355 nm); the laser time duration was 3nsec (full middle wide height), and the energy per laser shot was 5–20 μJ. The diameter of the impact varied from 10 to 30 μm. The depth eroded by each laser pulse was measured optically to within 2 μm; it varied from 20 to 30 μm for the first impact on the surface and from 30 to 50 μm for the following impacts in leaf depth. For each sample, 10 measurements were taken as a function of depth; the impacts on the surface were spaced along different axes at 100 μm intervals between impacts, on a total area between 1 and 2 mm^2. The mass spectra obtained at a given depth zone were cumulated for increasing the sensitivity of the method. For Zn, the absolute sensitivity was estimated to vary from 10^{-10} to 10^{-11}g with the biological material used. At the end of the analysis the laser craters were observed using a scanning electron microscope, type CAMECA MEB-07. The leaf surfaces treated with fungicides or $ZnCl_2$ were also viewed by scanning electron microscopy before and after the washing carried out with water or with N HCl, as described above.

Results

Cuticular retention in vitro. Zn retention by isolated pear leaf cuticles after a 24-hr immersion in fungicides or $ZnCl_2$ are reported in Table 2. The analysis of variance (F-test) showed a very significant effect of the chemical on the Zn retention. The distribution coefficient (λ) is expressed by the ratio of the Zn retained by the cuticles (in μg/g) to the Zn content of the immersion liquid (in μg/ml). λ was higher with fungicides than with $ZnCl_2$ (Table 2). Thus, cuticular retention of Zn was at least 5 times greater after a 24-hr immersion in fungicides than

Table 2. Zn retention by isolated pear leaf cuticles after a 24 hr immersion in fungicides or zinc chloride (0.2% w/v).

	Zn retention		
Fungicide	μg Zn/7 discs	μg Zn/mg cuticle	Coefficient of distribution[z]
Antracol	52.5	18.2	57.8
Calyram	8.9	3.2	25.1
M-Special	39.8	14.6	88.2
Polyram Combi	43.1	16.0	55.6
$ZnCl_2$	16.3	5.5	5.7
	Significance		
F-test	***	***	***
LSD (*t* for 5% level)	9.7	1.2	14.4

[z]The coefficient of distribution is expressed as the ratio: Zn retained by the cuticles (in μg/g cuticle) to the Zn content of the immersion liquid (in μg/ml). ***Significant at 0.1% level.

in $ZnCl_2$ solution, for the case of a single concentration (0.2% w/v) of chemicals, corresponding to different Zn concentrations.

Zn localization in situ. The observations obtained by scanning electron microscopy of the treated area of maize leaves showed that the fungicides deposited on the leaf surface were not completely removed by washing with water or N HCl. Residues were still visible, particularly after Polyram Combi and Antracol treatment (Fig. 1); the presence of Zn was measured using Si (Li) detector (Tracor System Model 880). The results of the Zn analysis as a function of the leaf depth are presented in Table 3. Zn was detectable only in the first surface zone corresponding to the level 0–30 μm with the 3 fungicides: Calyram, M-Special, and Polyram. Two mass spectra corresponding to a surface and an in-depth analysis are given in Fig. 2. Zn did not appear on the mass spectra in the case of the Antracol, and this was attributed to an analysis outside the limit of the deposit. Some elements appeared as surface contaminants (Al, Na) on the mass spectra.

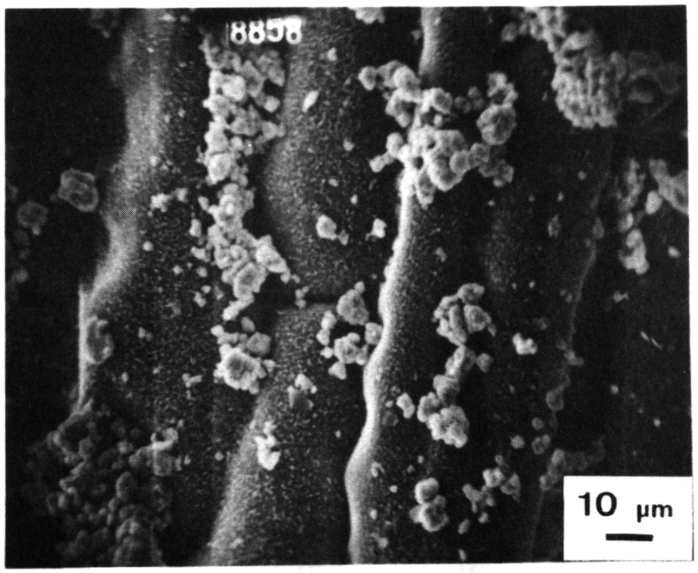

Fig. 1. Residues of Polyram Combi on a maize leaf after the washing of the treated part with N HCl.

Table 3. Analysis of Zn in the lamina of a maize leaf from the treated surface inwards.

Leaf depth[z] (μm)	Atomic relative units						
	Calyram			M Special		Polyram Combi	
	Analysis Axes			Analysis Axes		Analysis Axes	
	1	2	3	1	2	1	2
0– 30	0.68	0.82	6.44	0.08	ND[y]	1.18	0.80
30–160	ND	ND	ND	ND	ND	ND	ND

[z]Probed in increments of 30 μm; total leaf depth at point of analyses = 160 μm.
[y]ND = not detected.

Discussion

The results of the present work only concern the behavior of the metallic ion present in the fungicides; they are not necessarily applicable to the whole molecule. The experiment with isolated cuticles clearly demonstrate that the Zn contained in these 4 fungicides was retained at the cuticular barrier level. Retention could correspond to a fungicide sorption in the cuticular matrix or/and to the fixation of the metallic ion to the negative charges of the cuticular matrix, which is dependent on pH (8). The sorbed fraction is probably difficult to remove by washing because of the low solubility of these fungicides in water.

These results *in vitro* are consistent with the analysis carried out through the lamina thickness of maize leaves treated by the fungicides; Zn remained superficially located 24 hr after the foliar application. As the sensitivity of the method for Zn is about 10^{-10}g, it could be calculated that this detection limit represented 0.02 to 0.04% of the total Zn content of a droplet of 2 μl of fungicide at the concentration used here (0.2%). The Zn recovered in the treated leaf after a foliar application may correspond firstly to a fraction retained in the epicuticular waxes and not removed by washing as shown by scanning electron microscopy, and secondly to a fraction retained by the cuticular membrane. A third fraction might also be located deeper in the leaf, especially on the cell walls, since we have shown that Zn was only detectable in the zone 0–30 μm, which is much greater than the thickness of the maize leaf cuticle if we consider the values reported for different plant species, varying from 1 to 10 μm (7). This depth should be considered as a maximum limit for the detection of Zn because the method does not yet allow greater in-depth resolution with the plant material used. The techniques used in this study on fungicides for investigating the cuticular retention and the localization of an exogenous element in plant tissues may also be very useful for determining the fate of many other chemicals frequently deposited on leaves, such as nutrients, pollutants, and organic molecules.

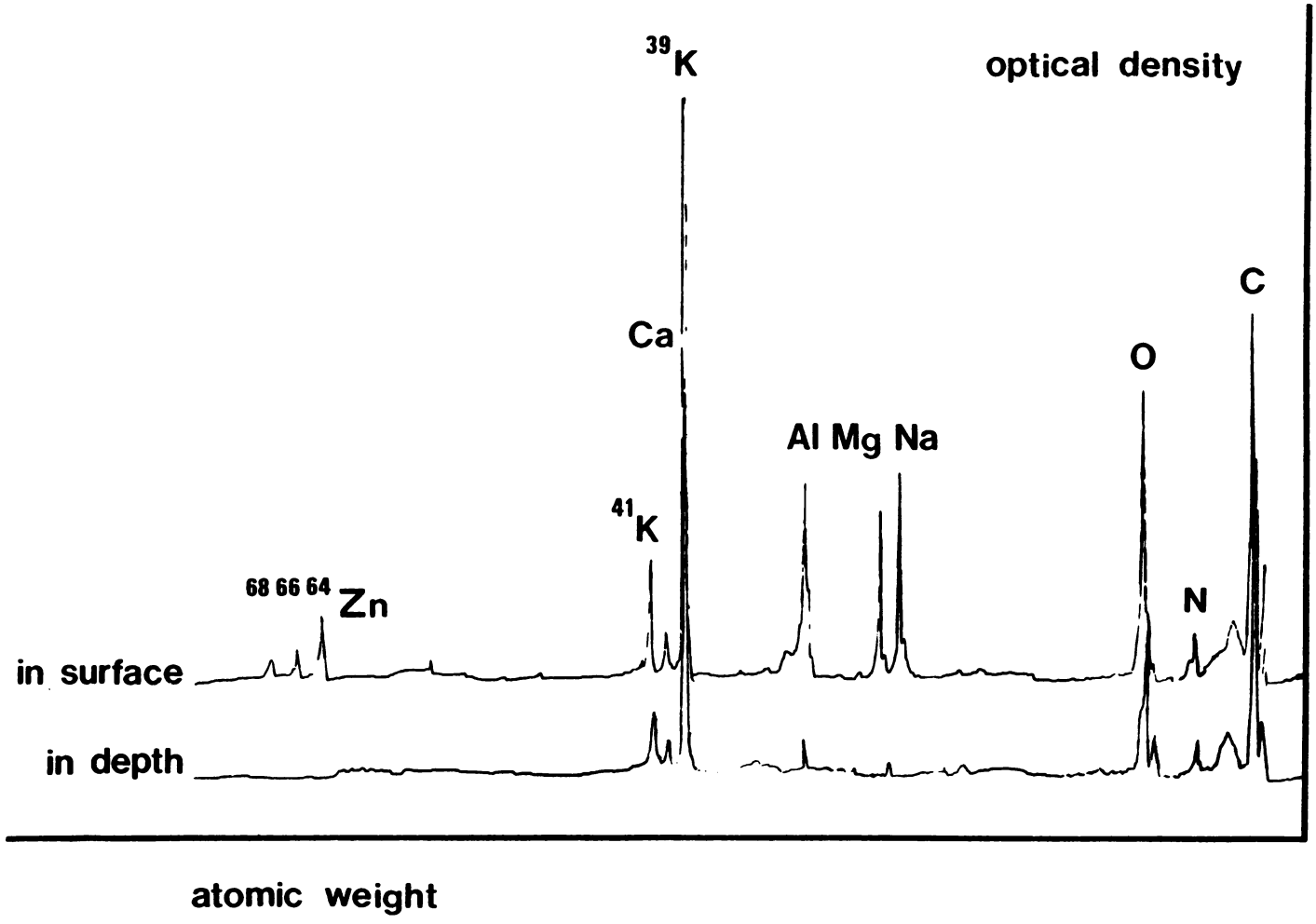

Fig. 2. Examples of mass spectra obtained with the laser probe mass spectrograph corresponding to a surface and an in-depth analysis of a maize leaf treated with Calyram.

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Differences in Biochemical Composition between 'Beurre d'Anjou' and 'Bosc' Pears during Fruit Development and Storage[1]

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Additional index words. Pyrus communis, ethylene, carbon dioxide

Abstract. **A comparative study in 1979 and 1980 between 'Anjou', a long-keeping winter pear and 'Bosc', a shorter keeping winter pear (both *Pyrus communis* L.) revealed that ethanol-insoluble matter, titratable acids, soluble solids, proteins, and free amino acids in fruit of both cultivars during fruit development, maturation, and storage period fluctuated from season to season and were not associated with their difference in postharvest life. Malic acid was the major fraction of organic acids in both cultivars, and it declined at a faster rate in 'Bosc' than in 'Anjou' during storage at −1.1°C. The amounts of citric, oxaloacetic, and fumaric acids were higher in 'Bosc' than in 'Anjou' and were maintained at constant levels throughout the storage period. Internal ethylene in both cultivars early in fruit development was about 0.3 ppm and decreased rapidly to below 0.07 ppm during late fruit development and harvest period. For 2 seasons, 'Bosc' was capable of ripening after less than 20 days of chilling at −1.1°C when its internal ethylene increased to 0.2 ppm, while 'Anjou' required at least 50 days of chilling to develop the ripening capacity coincident with an internal ethylene above 2.0 ppm. Internal ethylene accumulated in 'Bosc' about 8 times faster than in 'Anjou' during the first 60 days of storage at −1.1° and reached an equilibrium at 40 ppm for 'Bosc' and only 5 ppm for 'Anjou' during the remaining storage period. After any corresponding period of cold storage, both ethylene and CO_2 productions of 'Bosc' at ripening temperature of 20° were higher than those of 'Anjou', and 'Bosc' also required fewer days to reach the climacteric peaks than did 'Anjou'.**

During storage at −1.1°C, 'Bosc' pears have a much shorter postharvest life than 'Anjou' pears (i.e., 110–120 days for 'Bosc' and 180–220 days for 'Anjou') (11). The physiological basis for this difference is not known. Fruit acidity, soluble sugars, cell wall materials, and starch are common indicators of fruit maturity, quality, and storage life (5, 8, 9). Retention of organic acids in fruit stored in controlled atmospheres is always associated with a prolonged storage life (14, 16). 'Anjou' pears with high acid and sugar contents at harvest have a better postharvest quality than those with low acid and sugar contents, and higher amounts of proteins in 'Anjou' pears at harvest and during storage are associated with shorter storage life (18).

Ethylene ripens many climacteric fruits (20). Some fruits enter the climacteric soon after harvest, whereas others require a period of cold treatment to induce ripening capacity (4, 5, 15). Initiation of ripening is related to the threshold value of internal ethylene that accumulates in the fruit (10). In this study, we followed changes in various biochemical constituents, internal ethylene, and ripening capacity of these 2 cultivars from early in fruit development to late maturity, and during 5 months of cold storage in 2 consecutive seasons, in an effort to find the basic physiological difference(s) between the 2 cultivars so as to improve the storage and handling techniques.

Materials and Methods

Three uniform and mature trees of each cultivar were selected at the same orchard of the Mid-Columbia Experiment Station,

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