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Foliar absorption of pesticide in combination with adjuvants visualized through confocal laser scanning microscopy

Chime Mora-Garcia[1] and Pieter Spanoghe[2]
1- College of Arts and Sciences, Caraga State University, Ampayon, Butuan City, Philippines
2- Faculty of Bioscience Engineering, Universiteit Gent, Gent Belgium
chme03@yahoo.com
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ABSTRACT

Pesticide and the adjuvant can be taken up both by the plant and this can be determined using confocal laser scanning microscopy which can provide more evidence of the presence of pesticides in the plants. Oregon Green which has similar properties with some pesticides serves as the substitute since only fluorescent compounds can be visualized under the scanning microscope. Adjuvants bond, ethomeen T/25, silwet L-77, and softanol 70 were used in this study. The images obtained were observed and compared using Image J image analysis software. Images obtained from the confocal laser scanning microscope showed that silwet L-77 in combination with Oregon green was significantly different from the control indicating that penetration rate using silwet L-77 is faster allowing lower peaks during observation since most of the fluorescent dyes may have already diffused into the lower part of the leaf. Adjuvants bond, ethomeen T/25, and softanol 70 were not significantly different from the control suggesting that combination of adjuvants and pesticides will most likely cause an increase in uptake of pesticide into the leaf if the pesticide used is water soluble mixed with a hydrophilic adjuvant.

Keywords: Leaf uptake, adjuvants, Oregon Green, confocal laser scanning microscopy

1. Introduction

Modern agriculture would not have reached such high productivity without the development and usage of pesticides. These chemicals had improved food safety and quality, increase profit, and even improve human health. Due to this, pesticides will have to be used to guarantee that enough food supply will be generated for the next decades to come. However, pesticides create a problem to the environment since most of the chemical do not reach the target organism but are lost or contaminate the surroundings. To address the problem, adjuvants have been developed in order to improve the efficiency of pesticides needed to control the pest attacking the vital crops that have high demands in the market. Adjuvants are substances that can be added separately to a pesticide solution or substances that are included within the pesticide formulation to aid or modify the action of an agrochemical, or the physical properties of the mixture (Hazen, 2000). The study of adjuvants with pesticides is still relatively young (Green, 2000) but use of adjuvant results in a better weed and disease control and reduces the amount of active ingredient needed for the same control (Hazen, 2006). Presence of adjuvants in a spray mixture can disrupt the epicuticular waxes and enhance uptake of the active ingredient inside the leaf (MacIsaac et al., 1991). Efficiency of the pesticides in combination with adjuvants can be determined through its uptake in the plant leaves or roots. Uptake of such chemicals can be seen through visualization technique using microscopes which can provide evidence of the transport of pesticides in the plants.

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Visualization technique through the use of confocal laser scanning microscopy (CLSM) has been widely used and has become an important tool in plant cell biology (Hepler and Gunning, 1997). The three dimensional surface of an object is scanned point by point through a focused laser beam and creates the over-all picture by electronic means. Foliar absorption of pesticides and localization of chemicals within the leaf tissues and pathways by which diffusion occurs can be provided by CLSM (Liu, 2004). A CLSM is needed in studying the uptake of chemicals into plant leaves and to be able to visualize the difference in spread onto the leaf and penetration into the leaf between pesticides with and without adjuvants. However, very few studies were conducted using fluorescent microscopy such that limited information is available on the localization of pesticides in combination with adjuvants on leaf surfaces of plants, hence this study. This was undertaken to determine the foliar uptake and penetration of pesticide as influenced by adjuvants through the use of CLSM.

2. Material and methods

2.1 Confocal laser scanning microscopy

Uptake of pesticide was determined with the use of a CLSM. The use of this device became a very important tool for structural and dynamic analyses in plant cell biology (Hepler and Gunning, 1997). However, only fluorescent compounds can be visualized in the scanning microscope. Since pesticides are not fluorescent, a replacement chemical Oregon green was used. Oregon green has similar properties as herbicides in terms of molecular weight and polarity.

2.2 Adjuvants used.

Four different types of adjuvants namely bond, ethomeen T/25, silwet L-77, and softanol 70 were used in this study. Bond is a non-ionic co-polymer latex spreader sticker designed to improve spray deposition. Ethomeen T/25 is a tertiary alkyl amine ethoxylate, based on a primary tallow alkyl amine. It is an emulsifier in oil additive formulations which stabilizes an emulsion by increasing its kinetic stability. Silwet L-77 belongs to a silicone-based wetter/spreader group also known as organosilicones that contains a trisiloxane chain and this class has been increasing in popularity because of its superior spreading ability. Softanol is a non-ionic surfactant obtained by adding ethylene oxide (EO) and propylene oxide (PO) to linear secondary alcohols. It has extremely low foaming properties, low pour point but has excellent wetting characteristics.

2.3 Solution and Sample Preparation.

Oregon green ® 488 which has a commercial name 2', 7'-diflourescein was obtained from the company Invitrogen. Ten (10) mg of Oregon green was dissolved in a one liter volumetric flask obtaining a stock solution of 10 mg L^{-1}. The flask was covered with aluminum foil and kept in a cool and dark place since it tends to degrade in the presence of light. Different treatment solutions were prepared with 250 mg L^{-1} concentration each, and these are as follows; Oregon green serving as the control (T_0), Oregon Green + Ethomeen T/25 (T_1), Oregon Green + Bond (T_2), Oregon green + Silwet L-77 (T_3), and Oregon green + Softanol 70 (T_4). About five (5) ml of each solution were poured in its assigned petri dish. Bean (*Phaseolus vulgaris* L.) leaves were immersed in the solution for one minute and were replicated three times. Each of the leaves was taken out from the solution and was placed in

the oven chamber for an hour. After one hour, each of the leaves was washed with water for 2-3 minutes and blot dried by gently wiping the leaf with a tissue paper.

2.4 Preparation of slides.

Using the leaf cutter, a portion of the leaf was obtained and was placed over the glass slide with the upper layer facing upward. A droplet of oil immersion was added on the leaf before putting the cover glass. The oil immersion was important because the image would be distorted without it. Nail polish was used to fix the cover glass on the glass slide. When the nail polish was dry and the cover glass was not moving, the slide was ready for visualization under the scanning microscope.

2.5 Visualization of the leaf in CLSM.

Visualization of Oregon green with and without the adjuvants was done using a Nikon Eclipse TE300 epifluorescence microscope with a Biorad Radiance 2000 confocal system. The objective of the microscope was positioned below the glass holder hence an inverted type of microscope. The microscope objective used was a 60x water immersion objective and average duration of visualization was three minutes. The microscope was connected to a computer where digital images were obtained using Lasersharp 2000 program. Images were taken starting from the leaf surface up to a depth of 51 µm inside the leaf with sections every one µm. The images were observed and compared using the Image J image analysis software.

3. Result and conclusion

3.1 Oregon green without adjuvants

The study revealed the diffusion of Oregon green as influenced by adjuvants from the cuticle of the bean leaf into the epidermal cells. CLSM showed very thin layer of fluorescence in the cuticle and in the cytoplasm of the epidermal cells (Figure 1). Moderate amount of fluorescence can also be found in the epidermal guard cells as shown in the horizontal section image about 17 µm below the leaf surface. This indicates that diffusion and uptake of hyrodophilic compound such as Oregon green inside the leaf is slow and needs more time to penetrate deeper. Liu and Gaskin (2004) found that uptake of hydrophilic dye Oregon green was slow when applied to leaves with different leaf surface characters. Moreover, when surfactant was applied there was an increase in fluorescence intensity in the cuticle and the diffusion of the dye through the cuticle was also enhanced.

3.2 Adjuvant Bond.

Horizontal-section image of bean leaves showed that Oregon green was found in the epidermal guard cells but less fluorescence can be observed in the cytoplasm of the epidermal cells as reflected on the cross-section images (Figure 2). However, foliar uptake influenced by adjuvant bond has no significant difference (p value = 0.09) from Oregon green without the adjuvant even though the peaks observed with adjuvant bond were higher as compared with that of the control. This may imply that adjuvant bond may increase its penetration into the leaf but needs more time. The adjuvant bond, being a sticker, increases the adhesion of particles on target surfaces and may increase its penetration and uptake into the leaf better when it is mixed along with the compound used during application. Bond, being a sticker adjuvant aids in the attachment of the chemical to the leaf surface (Katagi, 2008)

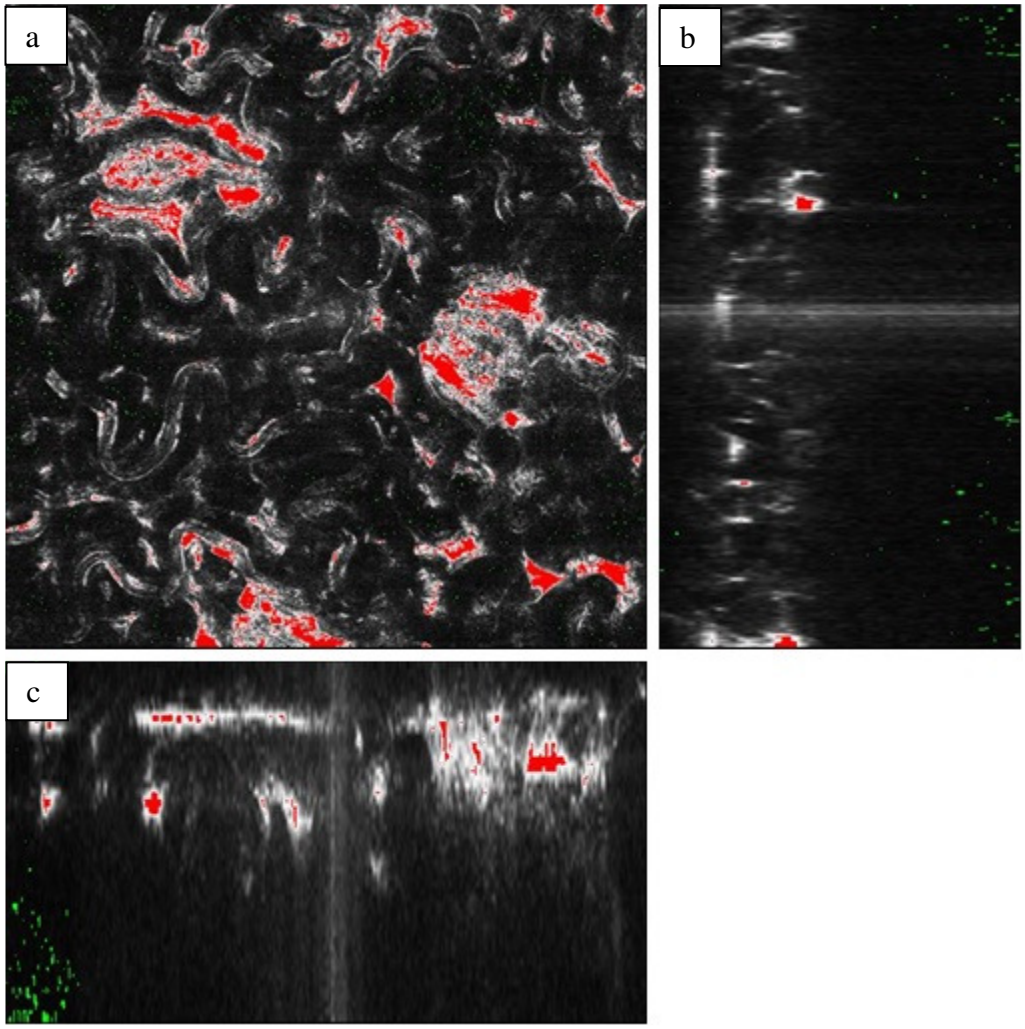

Figure 1. CLSM image showing localization of Oregon Green in bean leaf one hour after application (shown by the red color). (a) Horizontal section of the treated leaf ca. 17 µm below the surface; (b, c) Cross sections of the treated leaf.

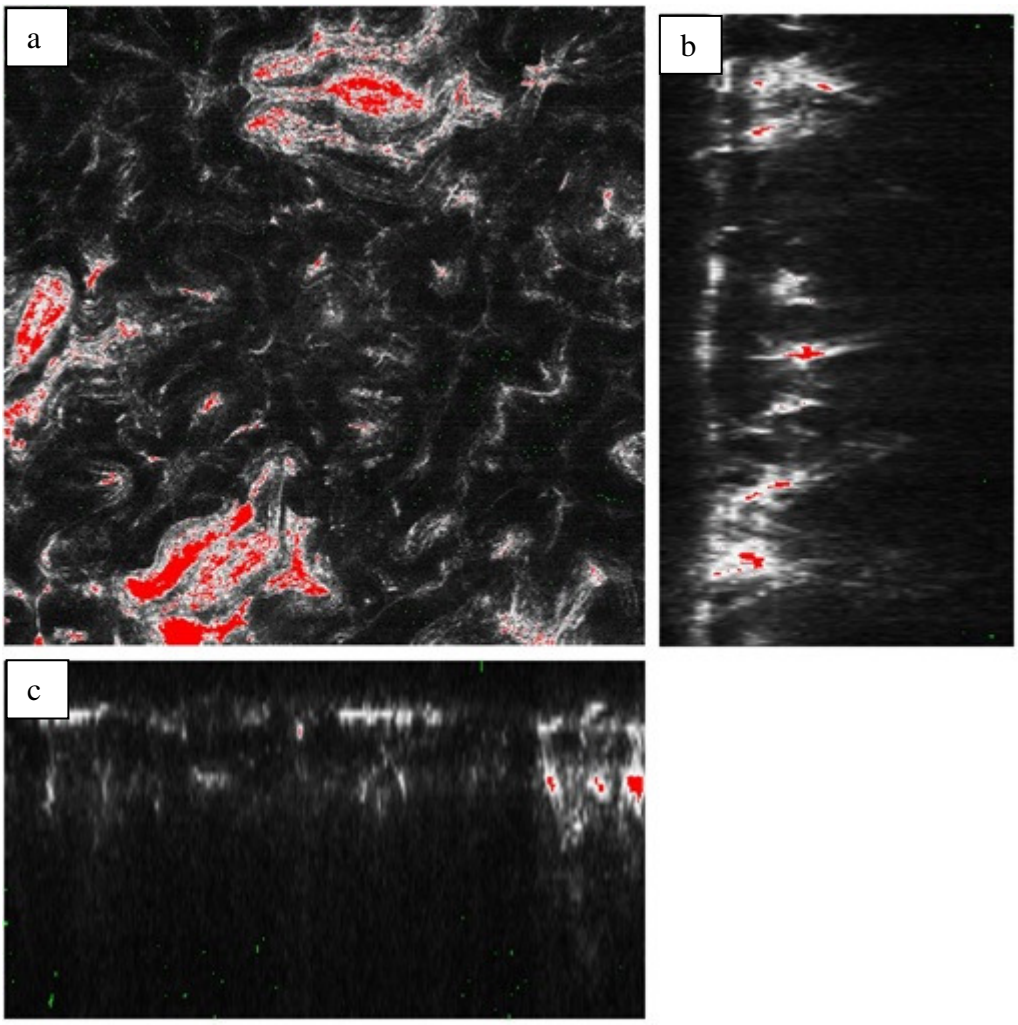

Figure 2. CLSM image showing localization of Oregon Green with bond in bean leaf one hour after application (shown by the red color). (a) Horizontal section of the treated leaf ca. 24 µm below the surface; (b, c) Cross sections of the treated leaf.

3.3 Adjuvant Ethomeen T/25.

Prominent fluorescent dye could be observed in the cytoplasm of the leaves as shown in the horizontal section image about 21 µm but less can be seen on the cross-sectional images (Figure 3). Uptake of the fluorescent dye did not differ significantly (p value = 0.92) from Oregon green without the adjuvant and peaks were observed to be almost the same with the control. This may indicate that with ethomeen T/25 present, the penetration rate of the compounds to the plant was not altered considering these compounds have the same properties as Oregon green. Ethomeen also has high hydrophile/lipophile balance (HLB) (14.7) which makes it hydrophilic. High HLB values demonstrated that hydrophilic adjuvants enhance the uptake of hydrophilic pesticides (Lui et al., 2004) which could be the reason why there was prominent fluorescent dye in the cytoplasm of the leaves.

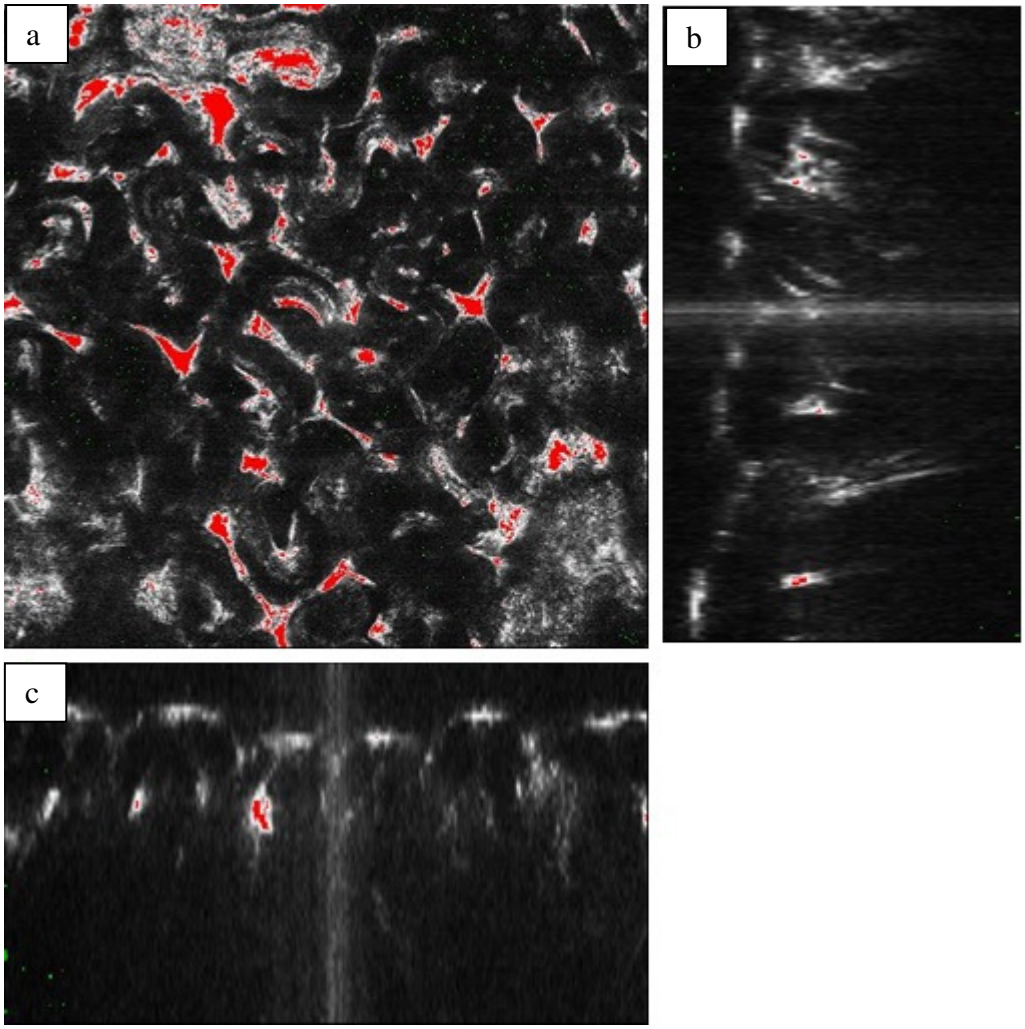

Figure 3. CLSM image showing localization of Oregon Green with ethomeen T/25 in bean leaf one hour after application (shown by the red color). (a) Horizontal section of the treated leaf ca. 21 µm below the surface; (b, c) Cross sections of the treated leaf.

3.4 Adjuvant Silwet L- 77.

The presence of the fluorescent dye in the horizontal section image was not very pronounced and only very weak fluorescence can be observed for both cross-sections (Figure 4). However, statistical results revealed that foliar uptake using silwet L-77 differs significantly from the control (p value = 0.004). Silwet L-77 is a superspreader which increases spray coverage on the leaf and may increase the penetration rate of the active ingredient into the leaf. Liu (2004) mentioned that presence of silicone surfactants such as silwet L-77 caused aqueous herbicides to flow into leaf tissues through stomata infiltration due to the low surface tension of the surfactant. This may have influenced the fluorescent dye to penetrate deeper into the leaf. Infiltration of pesticides into the leaves has been shown to be influenced by surface tension of organosilicones especially when surface tension value is less than 23mN/m (Katagi, 2008). This may indicate that penetration rate using silwet L-77 is faster allowing lower peaks during observation since most of the fluorescent dyes may have already diffused into the lower part of the leaf. Gaskin et al. (2000) also found significant differences between silwet L-77 and other adjuvants in terms of stomata infiltration and as concentration of adjuvants increased, infiltration into the leaves also increased.

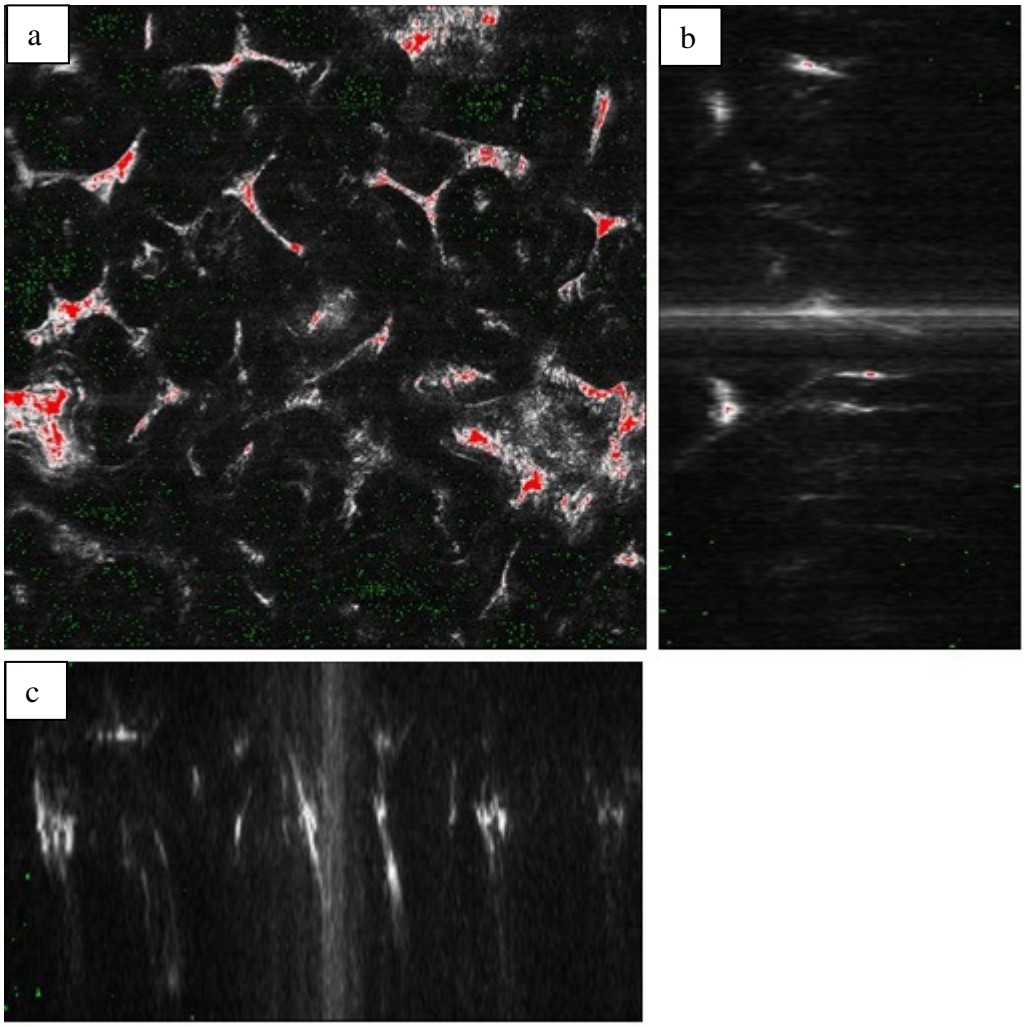

Figure 4. CLSM image showing localization of Oregon Green with silwet L-77 in bean leaf one hour after application (shown by the red color). (a) Horizontal section of the treated leaf ca. 25 µm below the surface; (b, c) Cross sections of the treated leaf.

3.5 Adjuvant Softanol 70

One (1) hour after application, an intermediate amount of Oregon green was found in the vacuole of the epidermal cells as shown in the horizontal section image about 19 µm below the leaf surface (Figure 5a). A small amount could also be observed in the vacuoles of epidermal cells as indicated in the cross-sectional images (Figure 5b and 5c). Foliar uptake of Oregon Green as influenced by softanol 70 did not differ significantly from the control (p value = 0.38) and peaks were observed to be lower than the control. Softanol 70 contains ethylene oxide and propylene oxide which are both hydrophilic and are attached to a linear alcohol surfactant which was found to be good uptake enhancers along with herbicides. A small amount of fluorescence observed in the vacuoles of epidermal cells could be due to the hydrophilic nature of the dye and the adjuvant added. Vacuoles are filled with water so that hydrophilic substances have a tendency to enter into this compartment. The use of non-ionic surfactants such as softanol was found to enhance spray deposition, adhesion, droplet coverage, and retention on the leaves (Yu et al., 2009).

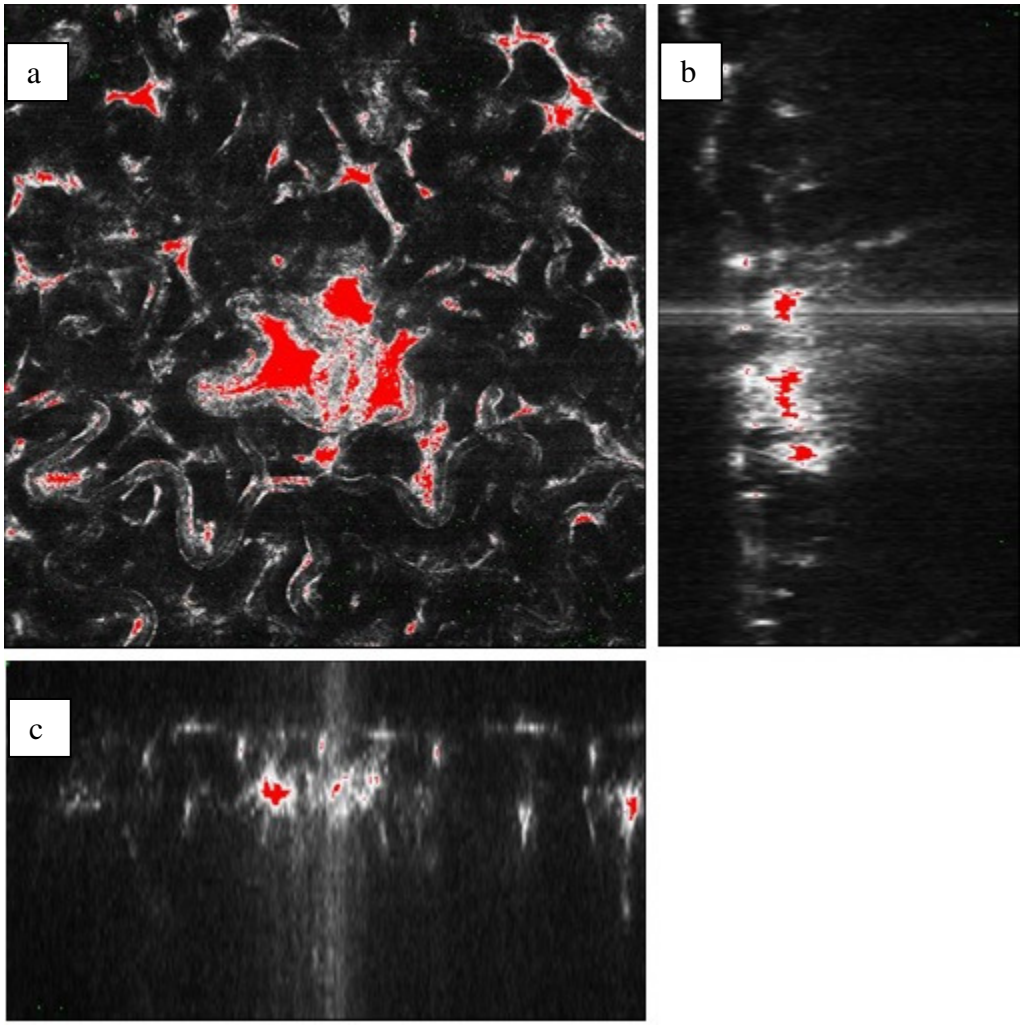

Figure 5: CLSM image showing localization of Oregon Green with softanol 70 in bean leaf one hour after application (shown by the red color). (a) Horizontal section of the treated leaf ca. 19 µm below the surface; (b, c) Cross sections of the treated leaf.

4. Conclusion

Images obtained from the confocal laser scanning microscope showed that silwet L-77 had lower peaks since fluorescent dyes were not prominent. However, it was only silwet L-77 in combination with Oregon green that was significantly different from the control. Silwet L-77 is a moderately hydrophilic adjuvant and this suggests that combination of adjuvants and pesticides will most likely cause an increase in uptake of pesticide into the leaf if the pesticide used is water soluble mixed with a hydrophilic adjuvant. Bond, ethomeen T/25, and softanol 70 however, were not significantly different from the control.

5. References

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