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Quantification of Sakuranetin in Paddy Leaves and Stem after Elicitation with Abiotic Elicitors (UV, $AgNO_3$, $CuSO_4$)

Mok Sam Lum (Corresponding author)

School of Sustainable Agriculture, Universiti Malaysia Sabah

Locked Bag 2073, 88999 Kota Kinabalu, Sabah, Malaysia

Tel: 60-88-320-000 E-mail: lmmoksam@ums.edu.my

Ratnavell Muniandy

School of Science and Technology, Universiti Malaysia Sabah

Locked Bag 2073, 88999 Kota Kinabalu, Sabah, Malaysia

Abstract

Phytoalexins are substances produced in appreciable amounts in plants only after stimulation of various biotic or abiotic agents. Sakuranetin was extracted from paddy leaves and stem after elicitated by silver nitrate, cuprum (II) sulfate and UV irradiation. Thin layer chromatography (TLC) was conducted to detect the presence of sakuranetin at R_f 0.09 under 365 nm UV light. Extracted sakuranetin was subjected to spectrophotometry at 337 nm. The concentration of sakuranetin present in the sample was calculated. Elicitation by silver nitrate significantly accumulated the highest amount of sakuranetin in leaves of paddy followed by UV radiation and cuprum sulfate. However, elicitation by UV radiation and silver nitrate in paddy stems produced significantly highest amount of sakuranetin. Comparing of sakuranetin amount recovered from leaves and stem, the leaves appeared to be significantly accumulated higher amount of sakuranetin than that recovered from stem.

Keywords: Sakuranetin, Phytoalexins, Abiotic elicitors

1. Introduction

Plants also defense themselves through production of secondary metabolites like phytoalexins. Phytoalexin is toxic antimicrobial substances produced in appreciable amounts in plants only after stimulation by various types of pytopathogenic microorganisms or by chemical or mechanical injury (Agrios, 2004). Phytoalexins are produced by healthy cells adjacent to localized damaged and necrotic cells in response to material diffusing from the damaged cells. Resistance occurs when one or more phytoalexins reach a concentration sufficient to restrict pathogen development (Lo *et al.*, 1996).

Phytoalexins like momilactones A and B (Cartwright *et al.*, 1981), oryzalexins A-F, oryzalexin S, phytocassanes A-D (Dillon *et al.*, 1997) and sakuranetin (Kodama *et al.*, 1992) can be found in diseases-infected paddy plants. Sakuranetin can be induced under a variety of biotic and abiotic stress stimuli. Sakuranetin showed the highest antifungal activity among the rice phytoalexins and the content of sakuranetin in blast infected rice leaves is much higher than that of other rice phytoalexins (Kodama *et al.*, 1992).

2. Materials and Methods

2.1 Plant material and growth conditions

The seeds of rice (Jaya variety) were germinated on cotton soaked in water in a small basin under laboratory temperature (25ºC) for six to ten days. The seedlings were then planted in sterilized organic soil. Basins without holes used to create flooded environment for optimum growing condition for paddy. Seedlings were grown under sun light (Dillon *et al.*, 1997).

2.2 Elicitation of sakuranetin using abiotic elicitors

2.2.1 Silver Nitrate ($AgNO_3$) Elicitation

Fresh leaves and stems of 50 eight weeks aged paddy plants were sprayed with 1% of $AgNO_3$ with a few drops of Tween 20. The plants were left for 3.5 days for phytoalexin accumulation.

2.2.2 Cuprum (II) sulfate ($CuSO_4$) elicitation

Fresh leaves and stems of 50 eight weeks aged paddy plants were sprayed with 5% of $CuSO_4$ with a few drops of Tween 20. The plants were left for 3.5 days for phytoalexin accumulation.

2.2.3 UV irradiation of Sakuranetin

About 15g of fresh leaves and stems of eight weeks aged paddy plants were cut and floated with adaxial side upper-most in tap water with a drop of Tween 20 in a clear plastic sandwich box. The box was then irradiated with UV light for 20 min. After irradiation, the box was sealed with cling film and incubated at 26°C under a 12 hours dark and 12 hours light regime, starting with a dark period of at least eight hours for 3.5 days (Dillon *et al.*, 1997).

2.3 Extraction of sakuranetin from rice leaves and stems

Fifteen-gram of each leaves and stem were cut into small pieces (1-2 cm). Leaf and stem pieces were soaked into 100 ml of 70 % aqueous Methanol (at boiling point) in a conical flask and boiled for 10 min on a hot water bath. After cooling, the flasks were left at room temperature for 24 hours in the dark. The extracts were then filtered through a Whatman No.1 filter paper and washed with 100ml of 70% aqueous methanol. The extracts were concentrated *in vacuo* at 40ºC. The concentrated leaf and stem extracts were re-extracted three times with 2 ml of diethyl ether. The combined ether fractions were evaporated to dryness on a rotary evaporator (Dillon *et al.*, 1997).

2.4 Detection of sakuranetin by using thin layer chromatography

Fifteen-milliliter of ethanol was added to the leaf and stem extracts. The extracts were spotted on thin layer chromatography (TLC) plate (Merck kiesel 60 F_{254}). The plates were developed in chromatography tanks pre-equilibrated with chloroform:ethanol (97:3 v/v) as developing solvent. Sakuranetin was detected at R_f 0.09 as a green fluorescence under ultraviolet light (365nm). Green fluorescence zone was marked under ultraviolet and scraped off into centrifuge tubes and eluted with chloroform:ethanol, 90:10 v/v. After filtering, the elute was concentrated to dryness (Atkinson & Blakeman, 1982).

2.5 Quantification of sakuranetin by spectrophotometry

The recovered sakuranetin was subjected to spectrophotometry with a maximum absorbance at 337 nm (Dillon *et al*., 1997). Using absorbtivity coefficient concentration of sakuranetin, the quantity of sakuranetin in sample was determined by Beer-Lambert Law.

3. Results

3.1 Elicitation of sakuranetin

After two days of elicitation with silver nitrate, cuprum sulfate and UV radiation, the leaves and stem of paddy started to show necrosis (Figure 1, Figure 2, Figure 3 and Figure4), whereas control leaves remained green for this period. These showed that a hypersensitive reaction had taken place.

3.2 Detection of sakuranetin by Thin Layer Chromatography (TLC)

All the extracts were developed in TLC plates to detect the presence of sakuranetin zone. Sakuranetin clearly can be seen under UV light at 365 nm with green fluorescence colour at R_f value 0.09 as in Figure 6 (Atkinson and Blakemen, 1982). Trace amount of Sakuranetin was also detected at R_f 0.09 in the control although the leaves and stems were not subjected to any chemical treatments (Figure 5).

3.3 Quantification of recovered sakuranetin

Sakuranetin recovered from TLC was subjected to spectrophotometry at 337nm for quantification. Graph from spectrophotometry for sakuranetin is shows maximum absorbance is 337 nm. Figure 7 illustrates the amount of sakuranetin accumulated in paddy leaves and stems after elicitation by UV radiation, silver nitrate and cuprum sulfate. Elicitation by silver nitrate significantly accumulated the highest amount of sakuranetin in leaves of paddy followed by elicitation by UV radiation and cuprum sulfate. However, elicitation by UV radiation and silver nitrate in paddy stems produced significantly highest amount of sakuranetin. Comparing sakuranetin amount recovered from both leaves and stem, leaves appeared to be significantly higher than that recovered from stem.

4. Discussion

Leaves and stem of paddy plants which already elicited by abiotic factors showed necrosis. High amount of sakuranetin can be extracted at the location from leaves that showed necrosis (Figure 1 & Figure 2). Previous study on *Ribes nigrum* leaves indicated sakuranetin was largely associated with the adaxial surface but low level of sakuranetin were found in abaxial surface extracts (Atkinson & Blakeman, 1982). They suggested that difference in structure of glands in both adaxial and abaxial sides might be the reasons for their different chemical contents. In addition, Dillon *et al*. (1997) suggested that adaxial surface of paddy leaves should receive UV irradiation in the methods. Tween 20 was used when silver nitrate and $CuSO_4$ were sprayed so that adhesion of the droplet on waxy leaves surface will give positive result.

Dark brown and chlorosis patches were observed on the leaves of elicited plant. Based on the observation, the dark brown patches were actually changed by the stained of abiotic agents and developed more rapidly than chlorosis. Chlorosis appears in later stages of elicitation due to structural change in the cell itself. Damage in cell such as swollen and disruption of chloroplast caused this yellowish lesion. Destruction of chloroplast decreased the photosynthesis rates and finally plants will die (Miyake *et al*., 2006). Elicitation of $AgNO_3$ also caused desiccation on protoplasm. Removal of water lead to the decreasing of protoplast volume that may itself have serious structural and metabolic consequences such as disruption in stem and leaves thus caused the plant wilted. Less dark brown lesion and chlorosis was observed in stem (Figure 3 & Figure 4) than leaves. It is because the surface of stem is covered by thick lignin compared to leaf that helps them to prevent from absorption of silver nitrate into the cell.

Elicitation by $AgNO_3$ significantly accumulated the highest amount of sakuranetin in paddy leaves followed by elicitation by $CuSO_4$ and UV irradiation. However, elicitation by UV irradiation and $AgNO_3$ in paddy stems produced significantly highest amount of sakuranetin. Leaves appeared to be significantly accumulated higher sakuranetin amount compare to stems (Figure 7).

The quantity of sakuranetin produced in stem part after $AgNO_3$ elicitation and UV irradiation was not significantly different (Figure 7). Thus, is probably due to the hard surface of the stem. The spraying might not be a suitable method for the $AgNO_3$ and $CuSO_4$ to be fully taken up into the stem. Thus, sakuranetin accumulation in the stem after elicited with abiotic agents was lower than in leaves.

There is no any research has been carried out before on paddy stem for sakuranetin accumulation. In this study, compared to leaves, stem accumulated lower amount of sakuranetin using all the three types of elicitation methods. According to Dillon *et al*. (1997), production of sakuranetin in paddy plant is a localized reaction. Compare to leaves, stem hardly produce lesions after elicitation. Therefore, fewer sakuranetin accumulated in stems.

Trace amount of sakuranetin was also detected at R_f 0.09 in the control, although the leaves were not subjected to any chemical treatments (Figure 5). The mean value of the sakuranetin produced in paddy leaves and stems were 0.11 μgml^{-1} and 0.09 μgml^{-1} respectively. However, the paddy leaves and stem elicited by $AgNO_3$, UV and $CuSO_4$ were accumulated higher amount of sakuranetin compared to control plates. It is known that biosynthesis of phytoalexins can be triggered by biotic and abiotic agents. Abiotic elicitors include chemicals, such as mercury salts and copper, physical agents such as injury, partial freezing and thawing, and UV radiation (Ebel, 1986; Soylu *et al.,* 2002). Thus, slightly production of sakuranetin in control might be due to the non biological stress, such as the rapid temperature changed outside our Lab.

Production of sakuranetin after elicitation by chemical elicitors like silver nitrate is higher compared to the non-chemical abiotic elicitors. The production of sakuranetin using chemical abiotic elicitors might not only caused by stress factor. Higher accumulation of sakuranetin may also due to either the release of plant's constitutive elicitors or the increased activities of the enzymes (Darvill & Albersheim, 1984) that could be intermediate in the elicitation of phytoalexin. Increase in the activities of enzymes have always been a reason for the production of phytoalexins and many researches had been done to investigate the degree of enzymes activities in conjunction with the phytoalexin synthesized (Hardviger & Schwochau, 1971; Loschke *et al*., 1983). This may also be applicable for sakuranetin as well.

5. Conclusion

Different amounts of sakuranentin were recovered from the extraction of elicited paddy leaves and stems. In this research, leaves produced higher amount of sakuranetin compared to stems. Comparison in the effectiveness of abiotic elicitors that have been used in this research, $AgNO_3$ is the best abiotic elicitors. $AgNO_3$ elicitation produced higher amount of sakuranetin followed by UV-irradiation and $CuSO_4$.

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Figure 1. Necrotic phases in paddy leaves after elicitation by $AgNO_3$

Figure 2. Necrotic phases in paddy leaves after elicitation by $CuSO_4$

Figure 3. Necrotic phases in paddy leaves after elicitation by UV irradiation

4(a) 4(b) 4(c)

Figure 4. Necrotic phases in paddy stem after elicitation by (a) $CuSO_4$, (b) $AgNO_3$ (c) UV irradiation

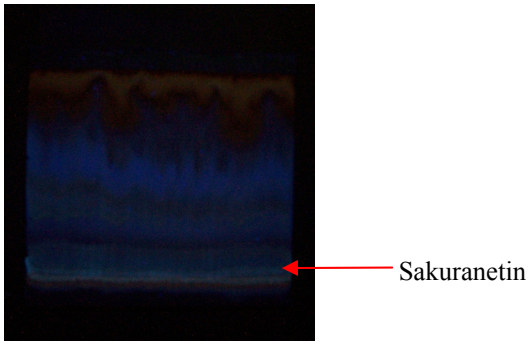

Figure 5. The presence of a trace amount of sakuranetin at R_f value 0.09 in control TLC plate for leaves under 365 nm UV light

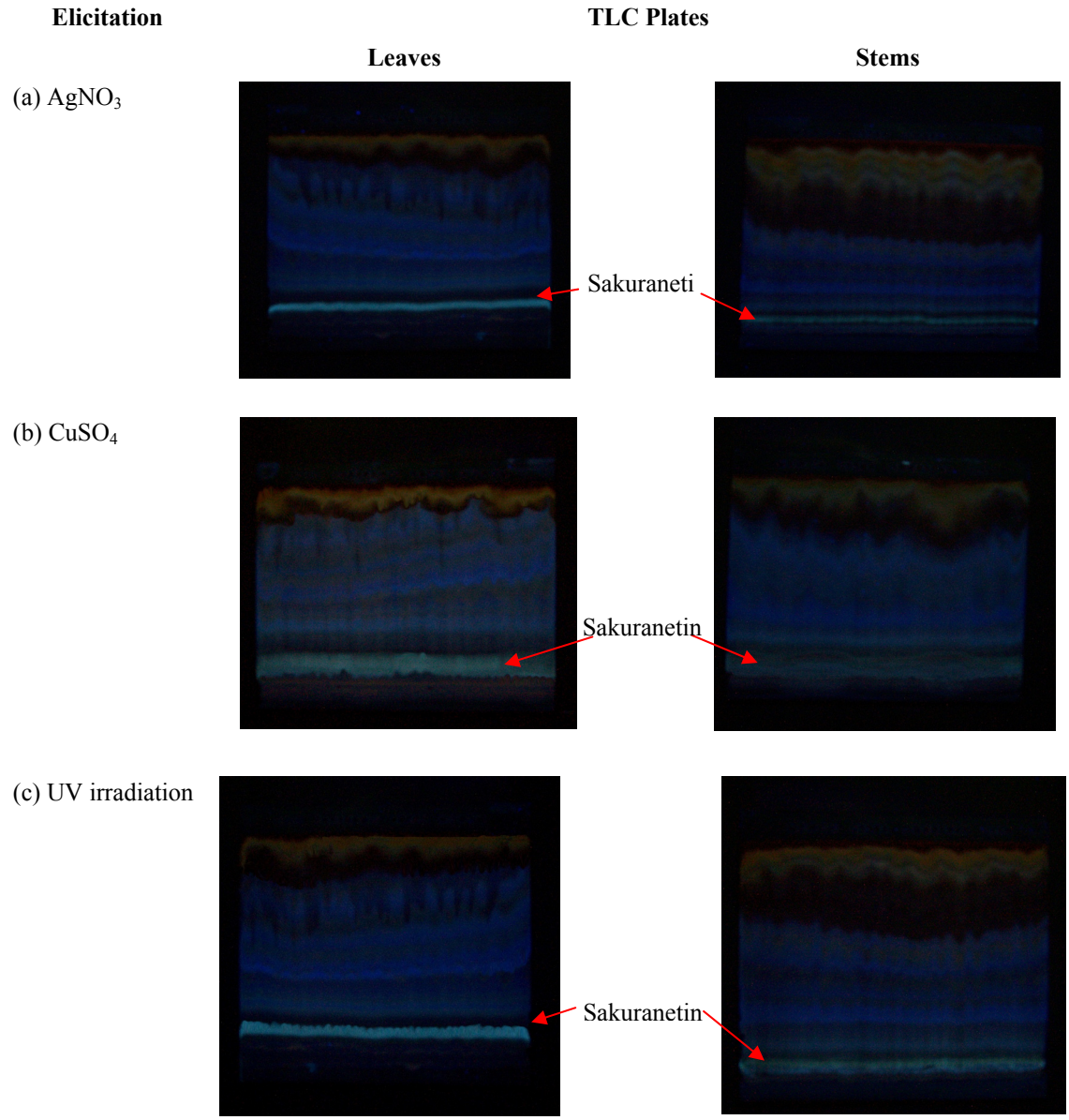

Figure 6. The presence of Sakuranetin at R_f value 0.09 in TLC plate under 365 nm UV light for leaves and stem subjected to $AgNO_3$ elicitation, $CuSO_4$ elicitation, and UV irradiation

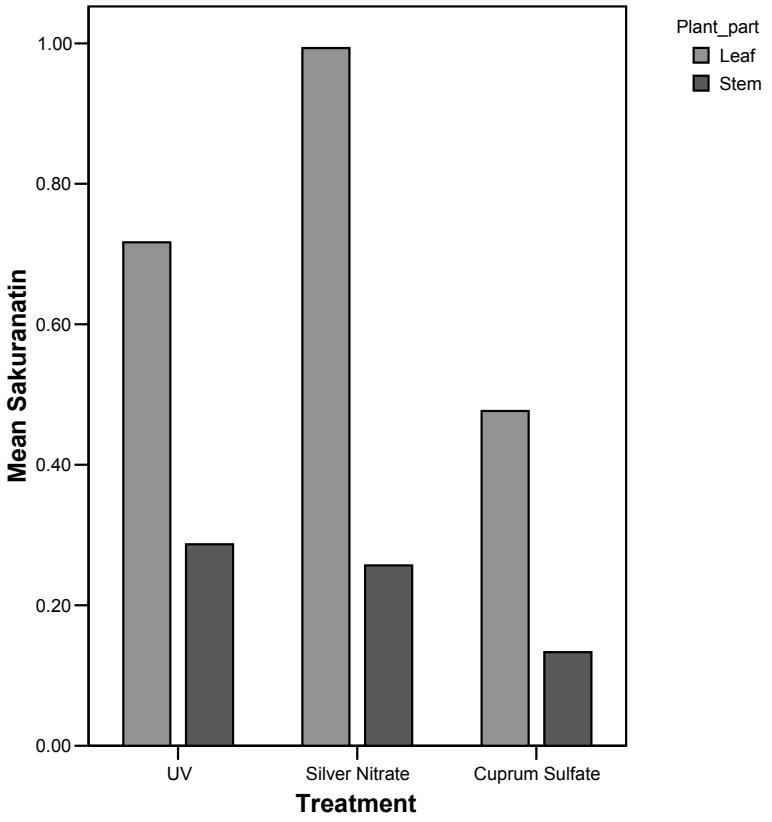

Figure 7. Sakuranetin recovered in paddy leaves and stem after elicitation by UV irradiation, silver nitrate and cuprum sulfate in bar chart