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Study of Optimum Mobile Phase for Determination of Phytoalexin in Rice by Thin Layer Chromatography

Pennapar Tansian[1, 2*] and Nonglak Parinthawong[1]

[1]Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok 10520, Thailand; [2] Center of Excellence on Agricultural Biotechnology: (AG-BIO/PERDO-CHE), Bangkok 10900, Thailand.

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Phytoalexins play crucial role in plant defense. These antimicrobial compounds are inducible and accumulated in plant after infection with biotic or abiotic stress. The highly accumulation of phytoalexin can be found in resistant plant varieties after induction compared to the susceptible varieties. In general, evaluation and characterization of the phytoalexin compounds are carried out using thin layer chromatography (TLC) method. However, mobile phase solvent is often limited the compound separation on TLC plate. Therefore, the aimed of this study was to select the optimum mobile phase for separating the extract of rice leaves for TLC. Four combinations of mobile phase included benzene : ethyl acetate (9:1), ethanol : chloroform (3:97), benzene : methanol (9:1) and benzene: methanol (1:9) were compared for best migration obtained on the TLC plate. Each mobile phase migration was done with 6 replications and the experiments were repeated 4 times. After comparing band appearance on TLC plate, the results showed that the combination of benzene : methanol (9:1) could separate 15 spots with clear R_f on TLC plate. This mobile phase combination will be used in further analysis on the characterization of induced resistance in rice plant.

Keywords: induced resistance, UV-radiation, rice leaves extraction

Introduction

Phytoalexin plays crucial role in plant defense. These low molecular weight antimicrobial compounds are inducible and accumulated in plant after treatment with biotic or abiotic stress. The interaction between plants and pathogens mostly depends on the ability of phytoalexin production in plants which they are exposed (Jeandet *et al*., 2014). The highly accumulation of phytoalexin can be found in resistant plant varieties compared to the susceptible varieties. Furthermore, phytoalexins accumulate around infection sites in case of infection with avirulent isolate of a pathogen but not after infection with virulent isolate (Conn *et al*., 1988). Fifteen phytoalexins in rice have been

* **Corresponding Author**: Pennapar Tansian; **E-mail address**: 58604047@kmitl.ac.th

isolated and characterized including 14 diterpenoid and 1flavonoid, sakuranetin (Grayer and Kokubun, 2001). The diterpenoid phytoalexins identified in rice were structurally classified into four groups: momilatone A and B, oryzalexin A–F, phytocassane A–E and oryzalexin S (Peters, 2006; Okada *et al*., 2007). The flavonoid sakuranetin was reported to exhibit fungicidal activity to pathogen and was highly accumulated in the leaves of rice plants after blast infection, suggesting that it plays an important role in the defense against pathogen in rice (Kodama *et al*., 1992). In general, evaluation and characterization of the phytoalexin compounds are carried out using thin layer chromatography (TLC) method. The selection of mobile phase is an important step, a good combination of mobile phase system selection is usually the most difficult part of TLC method. Combination of benzene: ethyl acetate (10:1, v/v) has been used as a mobile phase for separation rice extract on TLC plate which resulted in 4 phytoalexin spots with R_f values of 0.22, 0.28, 0.35 and 0.61. All 4 spots showed antifungal activity against pathogen (Kumar *et al*., 2006). The aimed of this research was to find the best combination of mobile phase for separating rice leaves extract using TLC method.

Materials and methods

Plant material

Rice (*Oryza sativa*) cultivar, Khao Dowk Mali 105 (KDML105) was used. Seeds were germinated in water for 4 days. Rice seedling were transfered to plastic tray (11 x 7 cells) filled with soil and fertilized (5 g of ammonium sulphate). Seedlings were let grown in greenhouse under natural light. The 21-day-old plants were used as plant materials.

UV treatment

The 21-day-old rice plants were treated with UV radiation (Actinic BL lamp (maximum emission at 600 nm, 18W UVA output, Philips Lighting Holding B.V., Poland) in dark growth chamber. The UV lamp were placed in growth chamber 80 cm above the rice plants and let irradiated for 2 h. The UV-irradiated plants were transferred to greenhouse and their leaves were collected 48 h after UV treatment. The untreated plants were used as control. The experiment of UV-treatment was repeated three times.

Extraction of rice leaves

Thirty-five grams fresh weight of leaves were cut into small pieces. Rice leaves were extracted twice with 350 ml of 70% methanol for 1 day, in the dark. The extract was then filtered through a Whatman No.1 filer paper. The extract was concentrated using vacuum at 40 ℃ until 8 ml of liquid remained. The extract was then transferred to a glass tube with screw cap. The concentrated extract was re-extracted four times with 5 ml of petroleum ether. The petroleum ether layer was collected in new glass tube and concentrated until 2 ml of liquid remained using rotary evaporator.

Comparison of mobile phases used in TLC method

Five times 1 µl each of leaves extract were spotted on silica gel TLC plate (60 F_{254}, Merck, US). The size of the TLC plate was 20 x 20cm. The plate was run in four mobile phases included benzene : ethyl acetate (9:1) (Kumar *et al*.,2006), ethanol : chloroform (3:97), benzene : methanol (9:1) and benzene : methanol (1:9). The experiment was repeated four times. The TLC plate was removed from the mobile phase when the front line of mobile solvent reached about 1 cm away from the top margin. The migrated compounds were determined under the UV lamp (blacklight blue, wave length 315-400 nm) and UV active spots were circle with pencil. The R_f value of UV active spot was calculated using the given equation. The migration analysis of all experiments was repeated 4 times.

The R_f value can be calculated as:

$$R_f = \frac{\text{distance spot travels}}{\text{distance mobile phase travels}}$$

Results

Extraction of rice leaves and Comparison of mobile phases

Two days after UV irradiation, 35 g of rice leaves were harvested and extracted in 350 ml of 70% methanol. The remaining about 8 ml of liquid was re-extracted and concentrated to dried. The final content of rice leaves extract from 3 UV-treated replications were 1.054, 0.700 and 1.428 mg/g fresh weight for replication 1, 2 and 3 respectively, and 3 replications of UV-untreated were 0.908, 3.291 and 0.322 mg/g fresh weight. Five times 1 µl each of rice leaves

extract was spotted on TLC plate and the extracts on the TLC plate were separated using four combinations of mobile phase.

The results showed that the combination of benzene : methanol (9:1) is the most optimum mobile phase for separating the extracts from rice leaves with clear spots and R_f on TLC plate. This mobile phase combination migrated 15 spots when the plate was let 1 h for migration. Combination of benzene : ethyl acetate (9:1) showed 8 spots on TLC plate with 1 h 40 min migration. Combination of ethanol : chloroform (3:97) separated the extract to 10 spots within about 1 hr migration (Figure 1). The R_f values in UV-untreated control and UV irradiation treatments were shown in Figure 2. While the extracts of neither UV-untreated control nor UV irradiation treatments could be separated when using the combination of benzene : methanol (1:9) as mobile phase.

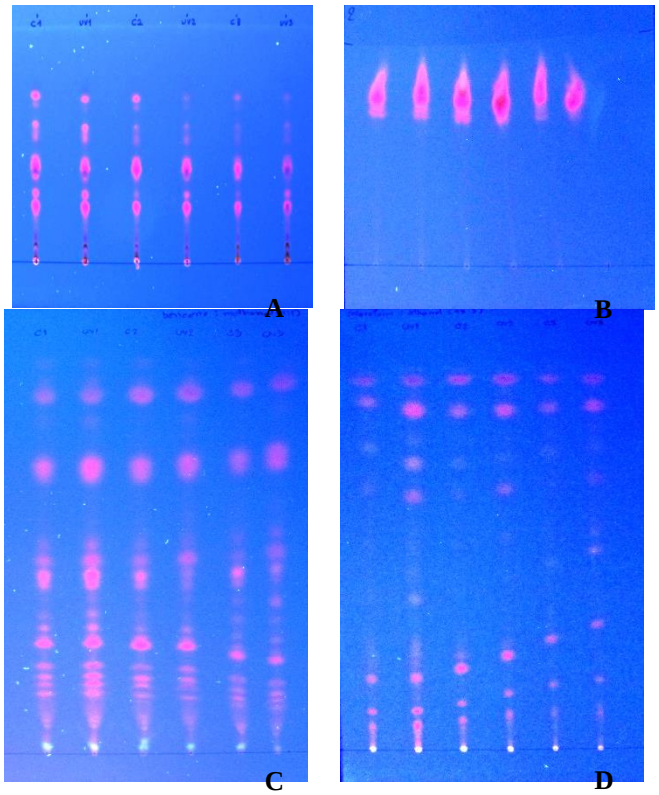

Figure 1 Migration patterns of rice leaves extract on TLC plate when using combination of benzene : ethyl acetate (9:1) (A), benzene : methanol (1:9) (B), benzene : methanol (9:1) (C) and ethanol : chloroform (3:97) (D), as mobile phase.

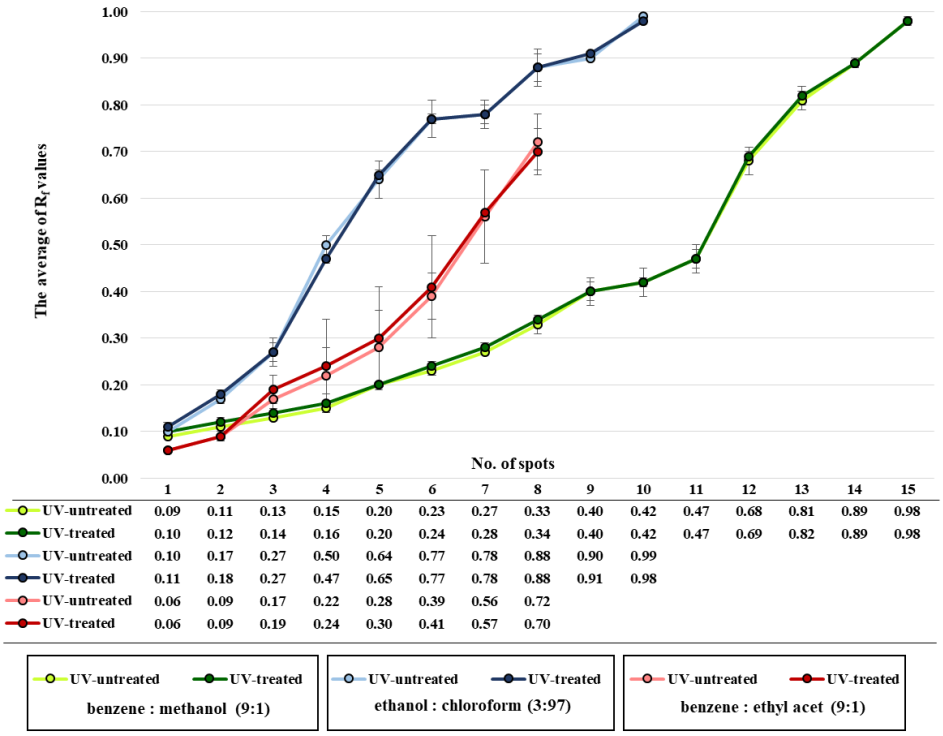

Figure 2. The average R_f values of rice leaves extract separated by 3 combinations of mobile phase.

Discussion

The most important step in separation of the plant extract by TLC method was the selection of suitable mobile phase that provide good migration. The key to succes of mobile phase selection depended on the polarity of both the plant extract compounds and the mobile phase solvents being used. When the extracts moved too fast and unseparatable from each other (Figure 1B), the polarity solvent should be used as mobile phase which the separation and migration was improved (Figure 1C). The selection of optimum mobile phase depend on number of spots that clearly appeared. In the good mobile phase, the R_f value should be repeatable in all replications and experiments (Figure 2). An important precaution was the using of stable chamber, once the mobile phase moved, the mobility of the mobile phase might be inaccurated. The great position of starting spot should be more than 1 cm away from the both left and right margins of the TLC plate. The difference between benzene : methanol (9:1) and benzene: ethyl acetate (9:1) was the polarity of solvents. Kumar *et al.*

(2006) used ethyl acetate as one of mobile phase in combination with benzene and the result showed different spots from our results on TLC because the polarity of ethyl acetate is lower than methanol. In this study, methanol was used as mobile phase therefore a highly polarity compounds obtained. In addition, methanol was well mixable with benzene. The results showed that methanol was better than ethyl acetate when using as mobile phase in combination with benzene for separating rice leaves extract with clear spots and R_f on TLC plate.

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