IRRIGATION, SPACING, AND SHADING ON LEAF AND ROOT GROWTH AND THE ACCUMULATION OF 3,7, 3'-TRIHYDROXY-4'-METHOXYFLAVONE AND STIGMASTEROL IN TUBEROUS ROOTS OF RED KWAO KRUA

Bunruam Khitka and Yuvadee Manakasem*

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Abstract

An experiment was conducted in Red Kwao Krua (*Butea superba* Roxb) at Suranaree University of Technology during 2002 - 2004. A split-split plot arrangement of treatments in a RCBD with 3 blocks was designed to study the effects of irrigation, spacing and light shading on leaf area, fresh and dry weight of the tuberous roots, and the photosynthesis rate of the Red Kwao Krua (RKK). The accumulations of stigmasterol and 3,7,3'-trihydroxy-4'-methoxy-flavone in the tuberous roots of RKK were also determined at the ages of 6, 8, 10, 12, and 14 months. The main plot treatments were irrigation at 3 day intervals, 7 day intervals, and rainfed condition as a control. The sub-plot treatments were spacing at 1.5 m × 1.5 m and 3 m × 3 m. The sub-sub plot treatments were shading at 70% and non-shading. The experiment was replicated 3 times. The spacing and shading did not have any effects on leaf area, tuberous root weight, and the photosynthetic rate at any plant ages studied. However, the irrigation regimes had an effect on the leaf area, tuberous roots weight, and photosynthesis rate of RKK at the ages of 12 and 14 months. The 3,7,3'-trihydroxy-4'-methoxyflavone was not found at any treatments and at any plant ages studied. However, the tuberous roots of RKK at the ages of 12 and 14 months that were irrigated at 3 day intervals and 7 day intervals accumulated stigmasterol at the level of 500 - 1,000 ppm. The irrigation regime was a significant factor increasing the amount of stigmasterol in the tuberous roots of RKK.

Keywords: *Butea superba* Roxb, cultivations, leaf and root growth, flavone, stigmasterol

Introduction

RKK is found in the forests of the north, the west and the northeast of Thailand (Wutythamawech, 1997). Thais have traditionally used it as a medicine for maintaining good health. The biochemical 3,7,3'-trihydroxy-4'-methoxyflavone accumulated in its tuberous roots probably maintain the sexual ability of men (Tocharus *et al.*, 2005). Many clinical nutrition products have this chemical as an additive. A large amount of crude extract of RKK has been exported to

School of Crop Production Technology, Institute of Agricultural Technology, Suranaree University of Technology, 111 University Avenue, Nakhon Ratchasima, 30000, Thailand. E-mail: yuvadee@g.sut.ac.th
** Corresponding author*

Germany, Japan and the USA (OARD, 2000). Stigmasterol, which has the same characteristics as b-sitosterol, can also be found with about 75% concentration in the roots (Dyas and Goad, 1987). These substances can be used by industry to synthesize steroid hormones. Stigmasterol is a natural product that has an effect on human sexual performance (Ryokkynen *et al.*, 2005). It is also used as a primary substance for birth control (Ryokkynen *et al.*, 2005). It is a popular product in clinical nutrition that helps in reducing the accumulation of cholesterol (Wongrattanasathit and Choomsri, 1992). With the need for the crude extract from RKK, research for producing RKK on a marketing scale can help prevent the removal of RKK from the forests. The effects of planting space, irrigation, and shading on the growth and accumulation of important chemicals in the tuberous roots of RKK were the focus of the research. The objective of this study was to investigate the effect of irrigation and shading on leaf area, the fresh and dry weight of the tuberous roots, and the accumulation of stigmasterol and 3,7,3.-trihydroxy-4'-methoxyflavone in the tuberous roots of RKK.

Materials and Methods

The lateral buds of RKK from Kalasin province were planted at the Suranaree University of Technology farm on January 1, 2003. A total of 180 regular 4 month old plants was selected and the plants were set as a split-split plot in a randomized complete block design (RCBD) with 3 blocks (replications). The main plots had irrigation regimes, namely non-irrigation, irrigation at 3 day intervals, and irrigation at 7 day intervals. The drip irrigation system was applied for 3 h in each treatment. The rain was considered as non-irrigation since every treatment got the same amount of water. The 2 sub-plots had plant spacing at 1.5 × 1.5 m and 3 × 3 m. The 2 sub-sub plots were non-shaded and 70% shaded, using 70% Salant covered at 0.5 m above the RKK. The main plots were treated when the RKK was 4 months old. The sub-plots were designed immediately at the planting time. The sub-sub plots were treated when the RKK was 6 months old. The leaf area, the fresh and dry weight of the tuberous roots, and the photosynthetic rate, including stigmasterol and 3,7,3'-trihydroxy-4'-methoxyflavone, were sampled and measured from the RKK at the ages of 6, 8, 10, 12, and 14 months.

Methods of Chemical Extraction and Determination

The tuberous roots of the RKK at the ages of 6, 8, 10, 12, and 14 months from each treatment were dug, cleaned, sliced into thin pieces, then dried at 55°C for 72 h, and ground to a powder. The extraction and determination of 3,7,3'-trihydroxy-4'-methoxyflavone were determined using methanol, chloroform:water, 80% methanol: Hexane, column chromatography and FT-IR (model spectrum GX, Perkin Elmer) according to the method of Ruksilp (1995), using 6 kg of the ground powder. The stigmasterol was determined using 10 gm (dry weight) per sample. The extraction method was done according to Ruksilp (1995). Then thin layer chromatography (TLC) by comparison with standardized stigmasterol (Sigma, St. Louis, MO, USA) was performed. The amount of stigmasterol was compared to the spot size of standardized stigmasterol (1 µl) at concentrations of 0, 100, 250, 500, 750, and 1,000 ppm, respectively.

Data Examination

1. Leaf area, fresh and dry weight of the tuberous roots, and the photosynthesis rate were examined from the RKK at the ages of 6, 8, 10, 12, and 14 months. Leaf area was monitored in mature leaf of 3 plants/replication by using a leaf area meter (Delta-T Image Analysis), and the photosynthesis rate was measured on 3 leaves/plant/replication by using a leaf chamber analysis type LCA-4 at 10.00 am - noon.

2. Examination of 3,7,3'-trihydroxy-4'-methoxyflavone from the extracted solution of the tuberous roots of the RKK from each treatment was performed by comparing the appearance of the important functional groups in the spectrum.

3. Examination of the concentration

of stigmasterol in the tuberous roots of the RKK from each treatment was evaluated by comparing the spot size with the standardized.

Data Collection

3,7,3'-trihydroxy-4'-methoxyflavone chromatography was performed on the solutions eluted with 5% methanol in chloroform. The infrared spectrum of the solution that was eluted with 5% methanol in chloroform was also collected. The amounts of 3,7,3'-trihydroxy-4'-methoxyflavone and stigmasterol were determined from each treatment and were statistically analyzed by analysis of variance (ANOVA) in a complete randomized design (Steel and Torrie, 1986). The significant differences between them were tested by Duncan's New Multiple Range Test (DMRT) according to the methods described by SAS (1985).

Results

Leaf and Root Growth

The spacing and shading did not have any effect on leaf area, the tuberous root weight, and photosynthetic rate at any ages of the RKK studied. However, the irrigation regimes had an effect on the leaf area, tuberous root weight, and photosynthesis rate of RKK at the ages of 12 and 14 months (Tables 1, 2, and 3). While the leaf area at the ages before 12 months old did not show any differences, this was because of the effect of precipitation in the rainy season at that time (Table 1). The dry weight per fresh weight of the tuberous roots of RKK was affected by the irrigation regimes (Table 2). The photosynthesis rate

Table 1. Effect of irrigation on leaf area (unit:cm^2)*

Irrigation regimes	6 months	8 months	10 months	12 months	14 months
Non irrigation	1,053.35	1,540.78	457.67	375.03 a	639.57 a
7 day intervals	1,333.98	1,642.06	574.12	551.84 b	1,472.49 b
3 day intervals	1,085.38	1,678.72	474.93	560.79 b	1,420.03 b

* *Within columns, means followed by the same letter are not significantly different according to DMRT (0.05).*

Table 2. Effect of irrigation on tuberous root dry weight/fresh weight (g/1g fresh weight)*

Irrigation regimes	6 months	8 months	10 months	12 months	14 months
Non irrigation	0.30	0.11	0.06	0.07 a	0.06 a
7 day intervals	0.34	0.11	0.06	0.04 b	0.03 b
3 day intervals	0.35	0.10	0.06	0.04 b	0.03 b

* *Within columns, means followed by the same letter are not significantly different according to DMRT (0.05).*

Table 3. Effect of irrigation on photosynthesis rate* (mmol CO_2 $m^{-2}s^{-1}$)

Irrigation regimes	6 months	8 months	10 months	12 months	14 months
Non irrigation	14.71	15.24	5.22	7.69 b	8.17 b
7 day intervals	14.28	14.19	4.81	15.84 a	18.21 a
3 day intervals	16.92	16.42	5.97	16.13 a	20.04 a

* *Within columns, means followed by the same letter are not significantly different according to DMRT (0.05).*

was affected by the irrigation regimes starting from 12 months old (Table 3). The RKK that was irrigated had a greater photosynthesis rate than that not irrigated by 2 fold or more. This has also been found in soybean and sun flower (Flavia, 1990).

Examination of 3, 7, 3'-trihydrohy-4'-methoxyflavone

The 3,7,3'-trihydrohy-4'-methoxyflavone did not accumulate in all samples studied. Each fraction eluted from the column with 5% methanol in chloroform was found to have the same components. Analysis via TLC using 20% methanol in chloroform as a mobile phase resulted in the materials that had retention mobility (R_f) of 0.70 (A) and 0.73 (B) mixed together in the same fraction. The polar reduction of the mobile phase for the TLC method from 20% methanol in chloroform to 10% methanol in chloroform could be used to separate these fractions. The two fractions (A and B) could be separated with the R_f values of 0.76 and 0.80 respectively. In order to obtain sufficient materials for structural analysis using infrared (IR) spectroscopy, the fractions remaining from the elution of 5% methanol were eluted through silica gel once more using 3% methanol in chloroform as the solvent. Finally, three fractions were obtained as follows (Figure 1):

1. The fractions that came out by TLC analysis using 20% methanol in chloroform as a mobile phase which had the R_f value of 0.73 (B).

2. The mixture of fractions which had the R_f value of 0.70 (A) and 0.73 (B).

3. The last fraction that came out had an R_f value of 0.7 (A).

Because 3,7,3'-trihydroxy-4'-methoxy flavone has an R_f value of 0.69, the last material that came out could be the chemical. To confirm the result IR spectroscopy was applied, as shown in Figures 2 and 3. The comparison of the IR spectroscopy of fractions A and B with the IR spectroscopy from the 3,7,3'-trihydroxy-4'-methoxyflavone standard showed that there were differences. The peak at a position of 1,650 cm^{-1} for the standard 3,7,3'-trihydroxy-4' methoxy-flavone (Ruksilp, 1995) did not appear on the spectrum of A and B [this peak is the specific character of the carbon group of the conjugated ketone (Srivibool, 1995)]. The absorption peaks at 1,739 cm^{-1} (Figure 2) and 1,715 cm^{-1} (Figure 3) were obtained in both A and B [these peaks are the specific character of the carbon group of the ketone (Srivibool, 1995)]. The results led to the conclusion that 3,7,3'-trihydroxy-4'-methoxy-flavone did not accumulate in these samples.

Examination of Stigmasterol

The amount of stigmasterol from each treatment was evaluated by comparing the spot size of the unknown material with that of standardized stigmasterol (Table 4). The only variable that resulted in a difference in the amount of the stigmasterol was the level of irrigation. The

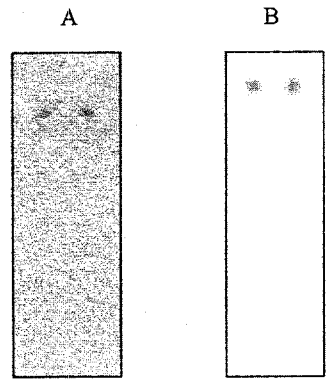

Figure 1. TLC chromatogram of substance A and B

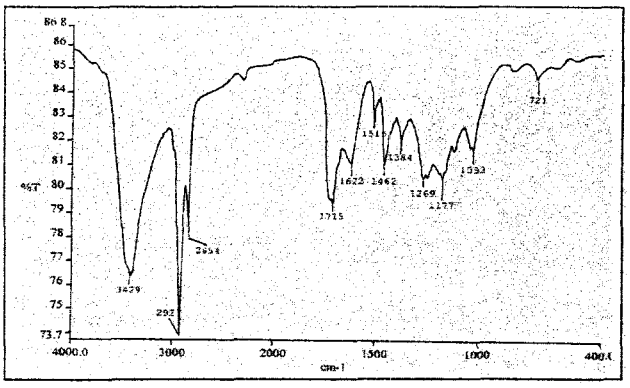

Figure 2. FT-IR spectrum of A

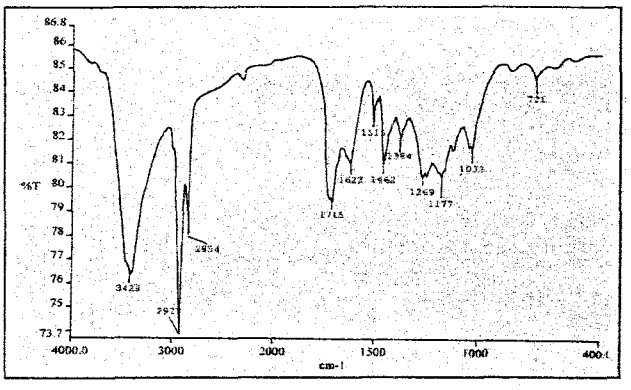

Figure 3. FT-IR spectrum of B

Table 4. The estimate by TLC of the concentration of stigmasterol in *Butea superba* Roxb*

Irrigation regimes	Spacing (m)	Shading	Concentration of stigmasterol (ppm)				
			6 months	8 months	10 months	12 months	14 months
non-irrigated	1.5 × 1.5	non	50	175	175 a	175 a	175 a
		shaded	50	175	175 a	175 a	175 a
	3 × 3	non	50	175	175 a	175 a	175 a
		shaded	50	175	175 a	175 a	175 a
7 day interval	1.5 × 1.5	non	50	175	375 b	625 b	875 b
		shaded	50	175	375 b	625 b	875 b
	3 × 3	non	50	175	375 b	625 b	875 b
		shaded	50	175	375 b	625 b	875 b
3 day interval	1.5 × 1.5	non	50	175	375 b	625 b	875 b
		shaded	50	175	375 b	625 b	875 b
	3 × 3	non	50	175	375 b	625 b	875 b
		shaded	50	175	375 b	625 b	875 b

** Within columns, means followed by the same letter are not significantly different according to DMRT (0.01).*

non-irrigated RKK had 50 ppm concentration of stigmasterol at the age of 6 months and 175 ppm at the age of 8, 10, 12, and 14 months (Table 4), while RKK that was irrigated at 3 and 7 day intervals had the same amount of stigmasterol. At the age of 6, 8, 10, 12, and 14 months the concentration of stigmasterol were 50 ppm, 175 ppm, 375 ppm, 625 ppm, and 875 ppm, respectively (Table 4).

Discussion

The irrigation regime was a significant factor increasing the amount of stigmasterol in the tuberous roots of RKK. It has been reported that RKK found in the forests in Lampang province accumulated 3,7,3'-trihydroxy-4'-methoxy-flavone; however, it was not the case in our study. This was possibly because the harvesting season could influence growth and development of the RKK, and it could also influence the synthesis of 3,7,3'-trihydroxy-4'-methoxy-flavone. The Doboysia in Australia accumulates much more hyoscine in summer than in winter (Luanratana and Griffin, 1980). The difference in the soil nutrition could affect the synthesis of the 3,7,3'-trihydroxy-4'-methoxyflavone substance. There has been a report that phosphorus and calcium could also stimulate the synthesis of hyoscine in Doboysia (Luanratana, 1992). The difference in the sea level between these two areas could also cause the accumulation of 3,7,3'-trihydroxy-4'-methoxyflavone. The different varieties of White Kwao Krua from 7 different locations that had differences in sea levels accumulated differening amounts of phytoestrogen (Ditchaiwong *et al.*, 2005). The difference in varieties could also have an effect on the synthesis of 3,7,3'-trihydroxy-4'-methoxyflavone. Finally, the difference in ages and the growth stages of the RKK could influence the synthesis and accumulation of the chemicals. For example, some plants synthesize anthocyanin when they are young to protect their shoots from excessive sun light (Fang and Hirsch, 1992). RKK in the forests in Lampang province that accumulated 3,7,3'-trihydroxy-4'-methoxy-flavone was probably older than the ones in this studied. The oldest in our study stet only 14 months old. The error could be caused from the extraction and separation steps. Further studies should improve these two steps to ensure that all of the stigmasterol is extracted from the samples. The use of gas chromatography or high-performance liquid chromatography probably can confirm the accuracy of the amount of stigmasterol (Abidi, 2001). The RKK that was irrigated at 3 and 7 day intervals had higher stigmasterol because it could photosynthesize more than that which was not irrigated (Sampetr, 1992). A higher level of photosynthate gives more substrate for synthesis of the chemical substances. This occurs in soybean grown at high temperature which has been irrigated and results in it having more oil than soybean that was not irrigated (Sampetr, 1992).

Conclusion

The spacing and shading did not have any effect on leaf area, tuberous root weight, and photosynthetic rate of RKK, for the ages studied. But the irrigation regimes did. Accumulation of 3, 7, 3'-trihydroxy-4'-methoxyflavone was not found at any irrigation levels and at any age of the RKK studied. However, the tuberous roots of the RKK at the ages of 12 and 14 months that were irrigated at 3 day intervals and 7 day intervals accumulated stigmasterol at the level of 500 - 1,000 ppm. The irrigation regime was a significant factor increasing the amount of stigmasterol in the tuberous roots of the RKK.

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