ANTHOCYANIN AND THE INFLUENCE OF NAA AND IRRIGATION ON POD AND SEED SETTING OF RED KWAO KRUA

Kesorn Muangtip and Yuvadee Manakasem*

Received: Mar 2, 2009; Revised: May 20, 2009; Accepted: May 25, 2009

Abstract

Red Kwao Krua (*Butea superba* Roxb.) is a protected medicinal plant of Thailand. The tuberous roots of Red Kwao Krua (RKK) are used to treat various illnesses and to maintain male hormones. Red substances are released when the tuberous roots of Red RKK are wounded. These substances were examined for an anthocyanin. The determination of the concentrations of an anthocyanin via TLC, absorbent wavelength, and pH differential techniques were performed on the root samples. The tuberous roots of RKK from the provinces Nakhon Ratchasima, Kalasin and Sakon Nakhon had anthocyanin concentrations from 69-144 μg/g fresh weight. RKK from Nakhon Ratchasima had the lowest an average of the amount of anthocyanin (96.80 ± 8.63 μg/g fresh weight). While RKK from Kalasin and Sakon Nakhon had an approximately the same amount of anthocyanin 112.27 ± 8.63 and 111.17 ± 8.63 μg/g fresh weight. However, RKK has difficulty in pod and seed setting. An experiment was conducted on RKK in Wangnumkeaw district, Nakhon Ratchasima from 2004-2005. The experiment was a 2^2 factorial in RCBD with 4 replications (1 replication had 10 subsamples). The effects of NAA and irrigation on pod and seed setting were examined. RKK that was irrigated and was sprayed with NAA at 100 ppm gave statistically significant differences in the highest the length of inflorescence (36.35 cm), the number of pods/inflorescence (5.10 pods/inflorescence) and the number of seeds/pod (1.15 seeds/pod). Therefore, NAA at 100 ppm plus irrigation could increase pod and seed setting. When using the stereomicroscopy to examine the seed, the sound seed showed a smooth seed coat, a big embryo, and was full of reserve food in the cell.

Keywords: *Butea superba* Roxb., anthocyanin, NAA, irrigation, pod and seed setting

Introduction

RKK is a protected plant of Thailand. Thais have traditionally used it as a medicine for maintaining good health. Red substances are released when the tuberous roots of RKK are wounded. The red substances could be an anthocyanin; anthocyanin has antioxidant activities. Brouillard and Laporte, (1977) tested for anthocyanin properties on red cabbage and found that the red solution from the red cabbage changed in response to pH. Franke *et al*. (2004)

School of crop Production Technology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand. Tel.:044- 224354, Fax.:044-224281, E-mail:yuvadee@sut.ac.th
* *Corresponding author*

found that anthocyanin had an absorbent wavelength between 510-540 nm. RKK has a very low pod and seed setting, which lends to a problem in its propagation. Medhi and Borbora (2002) found that spraying *Phaseolus vulgaris* L. with Nepthalene acetic acid (NAA) at concentration of 10 and 15 mg/L could increase its pod setting. Air temperature and soil moisture are the main factors for seed setting (Duangpatra, 1986). The objective of these studies was to examine the anthocyanin content and to investigate the effects of NAA and irrigation on pod and seed setting in the RKK.

Materials and Methods

Examination of Anthocyanin Resources of Experimental Plant

Tuberous roots of RKK were sampled from forests in the provinces of Nakhon Ratchasima, Kalasin, and Sakon Nakhon in the years 2004 to 2006. The determination of the concentrations of anthocyanin was done at Suranaree University of Technology.

Methods of Chemical Extraction and Determination of Anthocyanin

The Chemical Extraction of Anthocyanin

The tuberous roots were washed and peeled. After that they were separated into 3 parts: 1) the outer root coat (brown); 2) the cortex (red); and 3) the inner flesh (white). Then, only the cortex (red) part was examined for the amount of anthocyanin. This is because the red substances were come out from this part when the tuberous roots of RKK were wounded. The cortex was sliced into 2-3 mm pieces and 50 gm was grounded into powder. The extraction was performed using 150 ml of 1% hydrochloric acid (HCl) in methanol. The solution was in a flash closed with aluminum foil. Then the solution was shaker at 400 r/min for one hour. After that it was filtered with a thin white cloth. The residue was extracted again using 20 ml methanol. All of the extracted solutions were then centrifuged using a centrifuge (GS-15R-Beckman, Beckman Instruments Inc., Palo Alto, CA, USA) at 5,900 r/min. Then only the supernatant solutions were given the addition of 10% HCl in methanol at the ratio of 15 ml per 100 ml of extracted solution. The solutions were then refluxed at 90°C for an hour. The solvents were evaporated with a rotavapor (BuchiR-114, Buchi Labortechrik AG, Flawil, Switzerland) at 49°C until the extracted solutions were dried. The dry solutions were adjusted in volume by 20 ml methanol and were kept at -29°C (Nakamura *et al*. 1990; Wada and Ou 2002).

The Determination of the Amount of Anthocyanin

Thin layer chromatography (TLC) using 2 mobile phases of HCl:formic acid:water in the ratios of 7:51:42 and 25:24:51 (Sherma and Fried, 2003) was performed. The examination of anthocyanin using the absorbent wavelength of visible light at 400-700 nm and by comparing the R_f value of the standardized anthocyanin was investigated. Five ml of methanol was added to the solid of extracted solution that had the same R_f value of standardized solution. Then the solution was centrifuged at 5,900 r/min. The clear supernatant pink solution was used for the absorbent wavelength of a UV-Vis (using UV-visible/near infrared spectrophotometer). The changes of the color with the change of pH using the pH differential technique were also examined. HCl and Sodium hydroxide (NaOH) were used to adjust the pH from 1-4 and 5-13. The pH differential technique was also used to calculate the amount of anthocyanin by following the method of Wrolstad *et al.* (2005).

Investigation the Effect of NAA and Irrigation on Pod and Seed Setting

The experiment was conducted in Wangnumkeaw district, Nakhon Ratchasima from November 2004 to March 2005. The experiment was a 2^2 factorial in randomized complete block desing (RCBD) with 4 replications (1 replication had 10 subsamples). There were 2 factors: 1) NAA at 0 and at 100 ppm, and 2) not irrigation and irrigation (T1 = control –no NAA and no irrigation applied); (T2 = only irrigation applied); (T3 = only NAA at 100ppm applied); and (T4 = both NAA at 100 ppm + irrigation were applied). The RKK was sprayed 3 times on 24 November 2004, 15 December 2004 and 24 December 2004. The

irrigation was done at a daily interval from November 2004 untill March 2005.

Data Collection

The R_f values, the absorbent wavelength, and the amount of anthocyanin of the samples were collected. The number of pods per inflorescence, the length of inflorescence, the number of seeds per pod, and the weight of 100 seeds were also collected. The significant differences between them were tested using standard deviation (I = SD). The comparison of morphology between sound seed and unsound seed was also noticed by using the stereomicroscopy.

Results and Discussion

Anthocyanin

The cortex (red) crude extracted solution showed the R_f value in both mobile phases equal to the R_f value (0.34 and 0.12) (Figure 1) of anthocyanin. These R_f values are characteristic of anthocyanin (Sherma and Fried, 2003). Furthermore, this solution absorbed the wavelength of visible wavelength at the peak of 519 nm (Figure 2) which is similar to the absorption wavelength of anthocyanin (Longo and Vasapolla, 2006). Frank *et al*. (2004) found that anthocyanin has an absorbent wavelength between 510-540 nm (Figure 3).

In addition, this solution changed color from red to brown (Figure 4) when the pH was changed from 1 to 14. This is a characteristic of an anthocyanin (Figure 5) (Brouillard and Delaporte, 1977).

The tuberous roots of RKK from the provinces Nakhon Ratchasima, Kalasin, and Sakon Nakhon had anthocyanin at concentrations between 69-144μg/g fresh weights (Table 1). RKK from Nakhon Ratchasima contained the lowest the amount of anthocyanin (96.80± 8.63 μg/g fresh weight), while RKK from

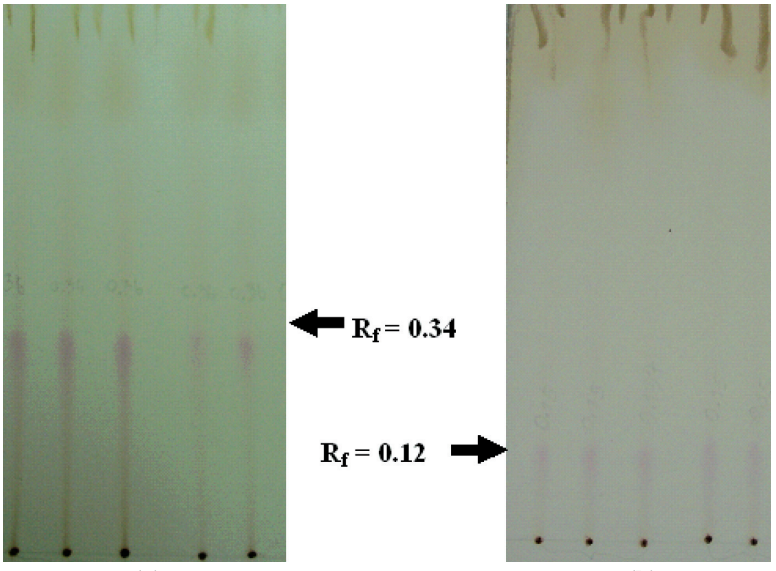

Figure 1. (a) TLC (HCl : HCOOH : H_2O = 7 : 51 : 42) of extracted solutions of the cortex showing an R_f value of 0.34
(b) TLC (HCl : HCOOH : H_2O = 25 : 24 : 51) of extracted solutions of the cortex showing an R_f value of 0.12

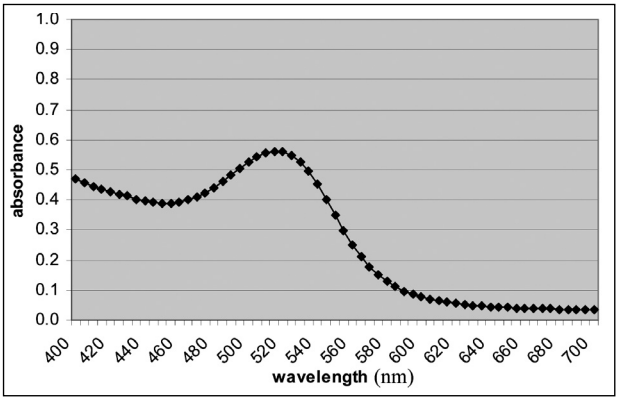

Figure 2. Spectrum of an extracted solution of anthocyanin from 400 to 700 nm (note the absorption at 519 nm)

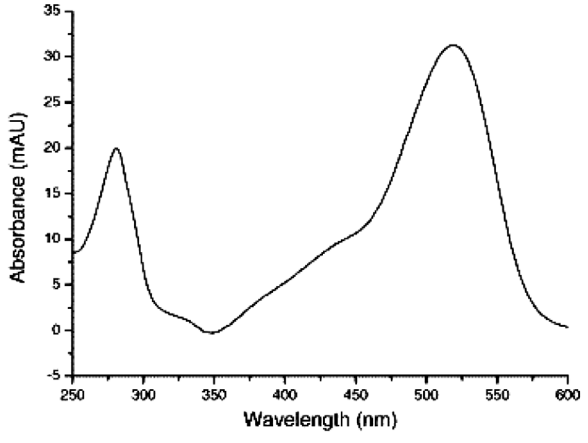

Figure 3. Standardized spectrum of anthocyanin (cyanidin) (Longo and Vasapollo, 2006)

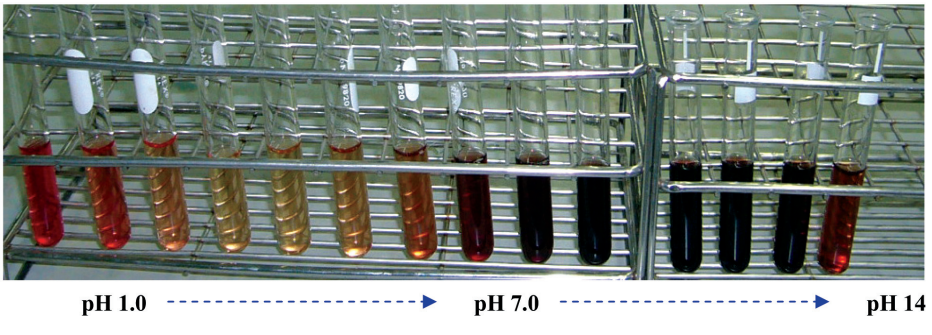

Figure 4. Colour changes of anthocyanin from pH 1.0 to pH 14

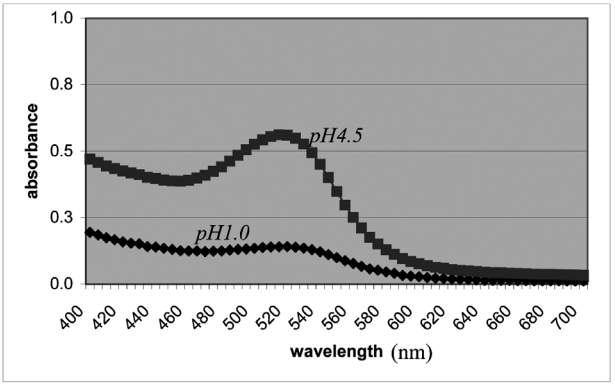

Figure 5. Spectrum of anthocyanin crude extracted solution at pH 1.0 and pH 4.5

Table 1. The amount of anthocyanin in the tuberous root of RKK from Nakhon Ratchasima, Kalsin, and Sakon Nakhon

Sample number	Sample sources	The amount of anthocyanin (μg/g fresh weight)	An average amount of anthocyanin (μg/g fresh weight)
N1	Nakhon Ratchasima	98	96.80 ± 8.63
N2		80	
N3		122	
N4		84	
N5		82	
N6		96	
N7		118	
N8		78	
N9		104	
N10		106	
K1	Kalasin	124	112.27 ± 8.63
K2		140	
K3		118	
K4		128	
K5		69	
K6		144	
K7		120	
K8		98	
K9		90	
K10		112	
K11		92	
SK1	Sakon Nakhon	81	111.17 ± 8.63
SK2		122	
SK3		82	
SK4		130	
SK5		132	
SK6		120	

Kalasin and Sakon Nakhon had approximately the same amount of anthocyanin (112.27±8.63 and 111.17±8.63 µg/g fresh weight). The differences in the concentrations of anthocyanin may be caused by the age of the plant, the environment that the plants grew, and/or the irregularity of the samples. There were any studies about an anthocyanin in the RKK before.

Pod and Seed Setting

The RKK that was sprayed with NAA at concentrations of 100 ppm plus was irrigated showed statistically significant differences in the length of inflorescence (36.35cm), the number of pods per inflorescence (5.10 pods) and the number of seeds per pod (1.15 seeds) (Figures 6, 7 and 8). These results implied that NAA at 100 ppm plus watering enhanced pod and seed setting. Ruhi *et al*. (2006) found that watering significantly lengthen the inflorescence of gladiolus. Tongumpai, (1994) reported that NAA and water can promote pod and seed setting in many kinds of beans. However, spraying the NAA at 100 ppm alone did not promote seed setting. Water is an essential factor for plants at the flowering and seed setting period (Kato, 1964; Westgate and Peterson 1993). None of the treatments showed statistically significant differences on the weight of 100 seeds. However, the treatments that were sprayed NAA at 100 ppm plus irrigated trended to have the highest 100 seed weight (146.80 gm) and the treatments that were only irrigated gave approximately the same 100 seed weight (146.75 gm) (Figure 9). Duangpattra (1986) implied that water is an importance factor to increase seed size and seed weight. Even though NAA at 100 ppm plus irrigation could increase pod and seed setting, the weight of 100 seeds

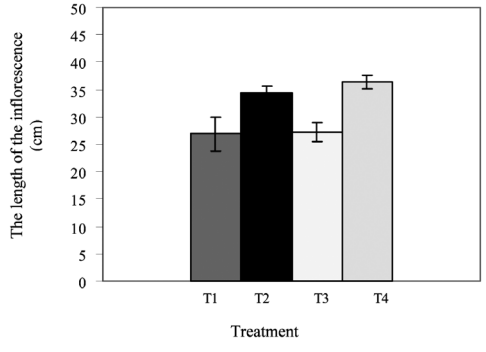

Figure 6. The length of inflorescence (I = SD)

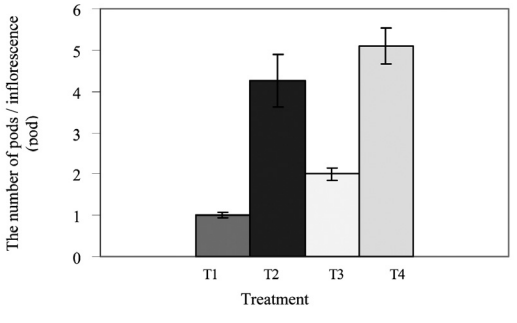

Figure 7. The number of pods per inflorescence (I = SD)

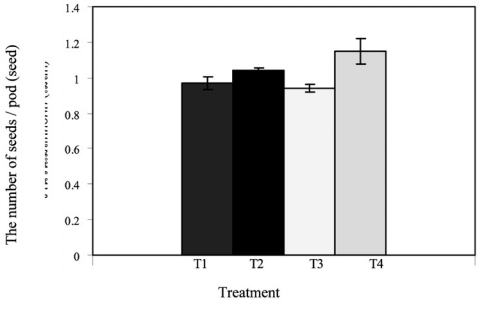

Figure 8. The number of seeds per pod (I = SD)

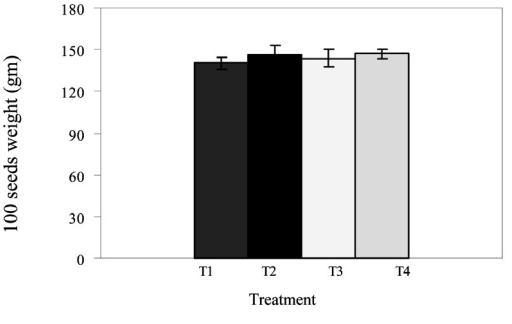

Figure 9. The 100 seeds weight (I = SD)

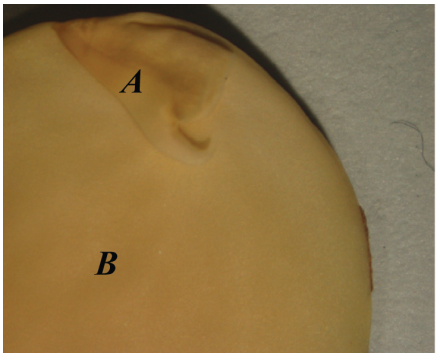

Figure 10. Sound seed
(A) Embryo, (B) Cotyledon

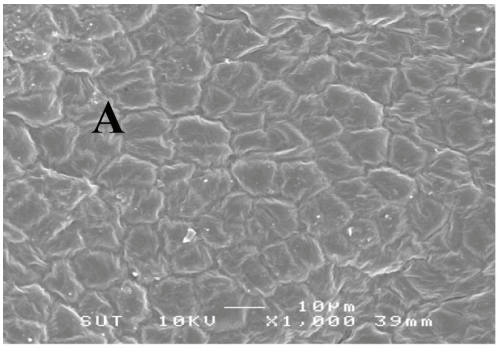

Figure 11. The cell of sound seed
(A) The cell that reserved food

were not statistically significant differences. This is because there were more partitioning of assimilate from photosynthesis to seeds and pods of those RKK that were not sprayed NAA and were not irrigated (which had less seed) than those RKK that were sprayed and were irrigated. Therefore, when 100 seeds were sampled to weight, their weight then showed none statistically significant differences. Further study on nutrition of RKK should be done. When using stereomicroscopy to examine the seeds of RKK, we found that the sound seed had a smooth seed coat and had a bigger size than the unsound seed. The cells of sound seed were also turgid and had bigger embryo than the unsound seed. The root tip was also healthy. The cells in the cotyledon were turgid, big, and healthy. They were full of reserve food (Figures 10 and 11), while the unsound seed showed the opposite results. The same results were found in White Kwao Krua (Chalardkid, 2003).

Conclusions

The tuberous roots of RKK contained anthocyanin. NAA at the concentration of 100 ppm and irrigation can increase pod setting. Seed setting could be promoted by irrigation. There were differences in morphology between sound and unsound seeds.

Acknowledgements

The authors acknowledge the assistance of Suranaree University of Technology and the National Research Council of Thailand (NRCT) for equipment and financial support.

References

Brouillard, R. and Delaporte, B. (1977). Chemistry of anthocyanin pigments. 2. Kinetic and thermodynamic study of proton transfer, hydration, and tautomeric reactions of malvidin 3-glucoside. J. Amer. Chem. Soc., 99(26):8,461-8,468.

Chalardkid, P. (2003). Influence of the environment and the other factors on vegetative growth, flowering, fruit setting and the accumulation of daidzein and genistein in the tuberous roots of White Kwao Krua [*Pueraria candollei* Grah. var. mirifica (Airy Shaw et Suvatabandhu) Niyomdham], [Ph.D thesis]. School of Crop Production Technology, Institute of Agriculture Technology, Suranaree University of Technology. Nakhon Ratchasima, Thailand, 98p.

Duangpatra, J. (1986). Seed Technology. Faculty of Agriculture, Kasetsart University. Bangkok, Thailand, 210p.

Franke, A.A., Custer, L.J., Ararkaki, C., and Murphy, S.P. (2004). Vitamin C and flavonoid levels of fruits and vegetables consumed in Hawaii. J. of Food Comp. Anal., 17:1-35.

Kato, I. (1964). Histological and embryological

studies on fallen flowers, pods and Abortive seeds in soybean, (*Glycine max* L.). Tokai-Kinki Nalt. Agric. Exp. Stn. Bull., 11:1-52.

Longo, L. and Vasapolla, G. (2006). Extraction and identification of anthocyanins from *Smilax aspera* L. berries. Food Chem., 94:226-231.

Medhi, A. K. and Borbora, T.K. (2002). Effect of growth regulators on the dry matter production, flower initiation and setting of French Bean (*Phaseolus Vulgaris* L.). Res. on Crops, 3(1):119-122

Nakamura, Y., Hidaka, M., Masaki, H. and Uozumi, T. (1990). Major anthocyanin of the flowers of Hibiscus (*Hibiscus rosa- sinensis* L.). Agriculture and Biological Chemistry, 54:3,345-3,346.

Ruhi, B., Osman, K.A., Koksal, A., and Dursun, B. (2006). The effects of drip irrigation on flowering and flower quality of glasshouse gladiolus plant. Agricultural Water Management, 81(1-2):132-144.

Sherma, J. and Fried, B. (2003). Handbook of the Thin-layer Chromatography. Marcel Dekker Inc., NY, 1,104 p.

Tongumpai, P. (1994). Plant Growth Regulators and Their Usefulness in Thailand. Faculty of Agriculture, Kasetsart University. Bangkok, Thailand, 196 p.

Wada, L. and Ou, B. (2002). Antioxidant activity and phenolic content of Oregon cane berries. Agric. Food Chem., 50:3,495-3,500.

Westgate, M.E. and Peterson, C.M. (1993). Flower and pod development in water-deficient soybeans (*Glycine max* L.Merr.). J. Exp. Bot., 258:109-117.

Wrolstad, R.E., Durst, R.W and Lee, J. (2005). Tracking color and pigment changes in anthocyanin products. Trends in Food Science and Technology, 16:423-428.