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Variation of neem seed morphology and azadirachtin content in different climatic zones of Thailand

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Abstract

Neem tree has high adaptability to a wide range of environmental condition and the variation in morphology and physiology is commonly found. Research on the variation of neem seed yields in terms of size, weight, oil and azadirachtin content in 4 different climatic zones covering 34 provinces of Thailand was carried out. The neem fruits were collected from collecting sites, cleaned, depulped and dried in the laboratory. Seed characters were measured, neem oil was extracted and azadirachtin content was determined by HPLC technique. The statistical analyses were performed to determine the variation in neem seed yield and its relationship with environmental factors. The size and weight of seeds among climatic zones was statistically significant. The mean annual rainfall and relative humidity had a highly positive influence on seed size, seed weight, oil content and azadirachtin content, meanwhile, latitude had a highly negative influence on those parameters. The highest azadirachtin content was found in neem seeds collected from tropical climate zone in Pattani province (2,028.63 mg/ml), and could be a suitable neem source for insect pest control program in the future.

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Introduction

Neem tree is a large semi - evergreen tree classified into the mahogany family, Meliaceae, and genus Azadirachta commonly found in South Asia and Southeast Asia (Schmutterer, 1990; ICIPE, 1995; Okonkwo, 2004). Generally, the neem tree grown in all regions of Thailand was composed of 3 species: *Azadirachta indica* var.siamensis. (Thai neem), wide natural distribution in Thailand and Southeast Asia, *Azadirachta indica* A. Juss. var. indica (Indian neem) and *Azadirachta excels* (Marrango tree), naturally found in western and southern Thailand (Willan *et al.*, 1990; Sanguanpong, 2000). All parts of the neem tree such as fruits, seeds, oil, resins, leaves, bark and roots has since ancient times been used for medicines, foods, furniture and pesticides. (Mohammed *et al.*, 2011; Jessinta *et al.*, 2014; Brahmachari, 2004).

In agriculture, the crude extract and oil from neem leaves and seeds were used as pesticide to control more than 400 species of insect pests, including many key crop pests and has proven to be one of the most promising plant extract for integrated pest management (Sahak *et al.*, 2010). The pesticide properties come from the presence of bioactive limonoids, such as azadirachtins, nimbin, salinin etc. (Kaushish *et al.*, 2006; Azam *et al.*, 2009). The significant active ingredient that mostly affect insects is tetranortriterpenoids called azadirachtin ($C_{36}H_{44}O_{16}$) (Butterworth and Morgan, 1968; Kavathekar, 2003; Donald *et al.*, 1992; Kraus, 1995) which is found mainly in the neem seed (Saxena, 1989; Kraus *et al.*, 1981; Kumar *et al.*, 1996).

Azadirachtin causes growth disruption, molting inhibition, egg - sterilization and other effects (Schmutterer, 1995; Koul *et al.*, 2004; Garcia *et al.*, 2006; Abdullah and Subramanian, 2008; Ware and Whitacre, 2004; Isman and Akhtar, 2007; Saxena, 1989). Azadirachtin also has hormonal effects, affecting both ecdysteroid and juvenile hormone (Garcia *et al.*, 2006; Abdullah and Subramanian, 2008; Morgan, 2009). Saxena and Tikku (19 90) reported that azadirachtin acts as insect repellant, deter feeding and oviposition, as well as inhibit growth and reproduction.

Neem products is a natural source of pesticide and it is classified as green technology that is eco - friendly with natural degradation, non - toxic to humans and animals (Brahmachari, 2004; Koul *et al.*, 1990) and have no residual effect on agricultural products (Ogbuewu *et al.*, 2011). Neem based pesticides are easy to prepare by extraction of seeds collected from naturally grown neem trees. (Brahmachari, 2004). Neem tree has high adaptability to a wide range of environmental conditions: high mountain, coastal area, rain forest, dry area (Sateesh, 1998). Neem tree requires little water and plenty of sunlight (Sateesh, 1998; Rengasamy *et al.*, 1993; Sidhu *et al.*, 2003).The variation in morphology and physiology of neem tree on oil and azadirachtin content have been reported (Singh, 1987; Rengasamy *et al.*, 1993; Gupta *et al.*, 1998; Sidhu *et al.*, 2003; Momchilova *et al.*, 2007; Demirbas, 2009), although the species of the plant are the same (Goja, 2013).

Thailand is located around the tropical zone near the equator. Each region has a different temperature, humidity, rainfall, and soil conditions (Sanguanpong, 2000). However, the variation of neem seed yield in terms of size, weight, oil content and azadirachtin content in different climatic zones of Thailand has not been reported. Therefore, this study was undertaken to quantify the variation among different zones and their correlation with the environmental conditions of the 4 different climatic zones of Thailand.

Materials and methods

Neem seed fruits collection from neem sites

Collection of neem fruits were carried out during harvest season from May to July 2017, covering 34 provinces in 6 regions of Thailand: Northern (Chiang Mai, Chiang Rai, Phayao,Uttaradit), Central (Bangkok, Samut Sakhon, Samut Songkhram, Nakhon Pathom, Suphan Buri, Chai Nat, Sing Buri, Ayutthaya, Lop Buri, Uthai Thani, Nakhon Sawan, Kamphaeng Phet), Northeastern (Khon Kaen, Nakhon Ratchasima, Ubon Ratchathani, Maha

Sarakham, Mukdahan), Eastern (Chachoengsao, Chon Buri, Rayong, Sa Kaeo), Western (Prachuap Khirikhan, Phetchaburi, Kanchanaburi, Ratchaburi) and Southern (Surat Thani, Phatthalung, Chumphon, Songkhla, Pattani) regions (Fig. 1).

Fig. 1. The location map of samples collected in 34 provinces in 6 regions throughout Thailand.

Only 5 kilograms of ripe neem fruits, greenish - yellow to yellowish in color, were collected from 5 neem sites in each province, mixed up to 25 kilograms/province and brought to the laboratory. The fruit samples were washed and depulped to get neem seed and then shade dried at 60 °C for 72 hours. The total of 1 kilogram of dried seeds were randomly selected for further study. Seed morphology such as color, weight and size were measured by standard procedure from 100 seeds. The dried sample seeds were decorticated, ground and sifted with a 100 mesh sieve and neem powder was processed for oil and azadirachtin content.

Neem oil extraction procedure

The dried seed powder (100 g) from each sample were placed in thimble and extracted in 150 mL of n – hexane in a Soxhlet apparatus at 40°C for 24 hrs. The obtained extract was concentrated in a rotary evaporator under vacuum to remove all the volatile solvents. The extraction was done in three replications. The color and amount of oil extract were determined and the percentage of neem oil was calculated on a seed weight basis.

Analysis of azadirachtin content by HPLC technique

Fine powder neem seeds from each province at 50 grams was extracted in 500 mL of methanol using a stirrer at 1,000 rpm for 12 hrs and incubated for 24 hrs. The extract was filtered using muslin cotton and filter paper (Whatman. No.1). All the volatile solvent was removed in a rotary evaporator under vacuum. The dark brown remain was dissolved in methanol and only 20 ml was utilized in quantifying

azadirachtin using HPLC. Phenomenex (USA) C 18 columns (150 x 4.6 mm.) with guard column was used in the mobile phase of water, acetonitrile and methanol in the ratio 50 : 35 : 15 with a flow rate of 1 ml/min and eluted samples were detected at 215 nm. Quantification of the azadirachtin was based on injections of known quantities of standard samples, Azadirachtin (Sigma Aldrich, USA) (95 % pure).

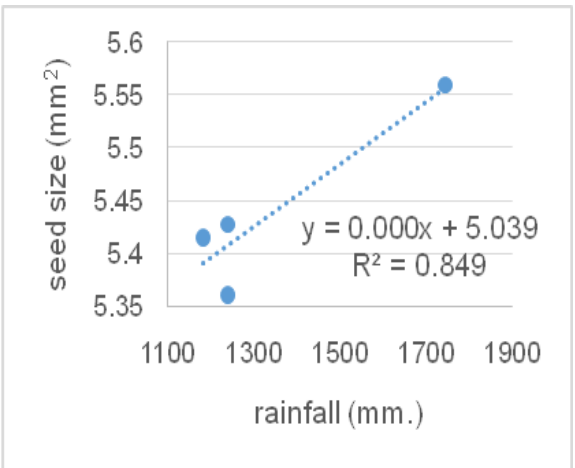
Data Analysis

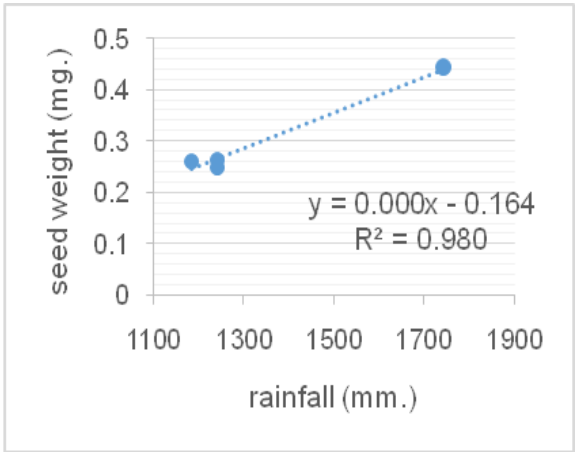
The morphological data of neem fruit, oil and azadirachtin contents from all provinces were arranged and modified into 4 groups based on information of climatic zones obtained from Meteorological Department of Thailand and analyzed using analysis of variances (ANOVA) and Duncan Multiple Range Test (DMRT) at 5 % significance level based on program R. version 3.5.0 (R Core Team, 2018).

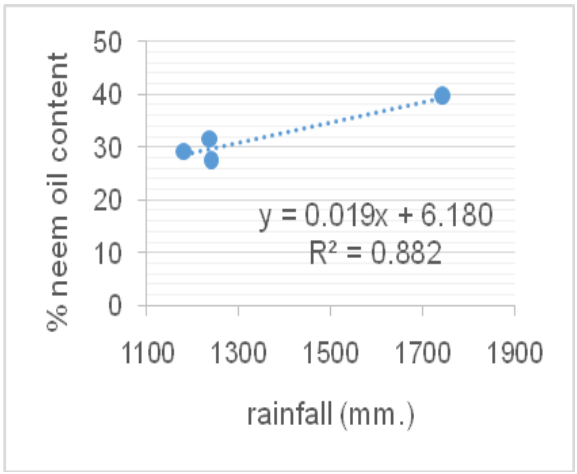
Correlation and regression analyses were employed to find the relationship between the meteorological data with the oil and azadirachtin contents. The appropriate neem source in terms of oil and azadirachtin content was screened and selected.

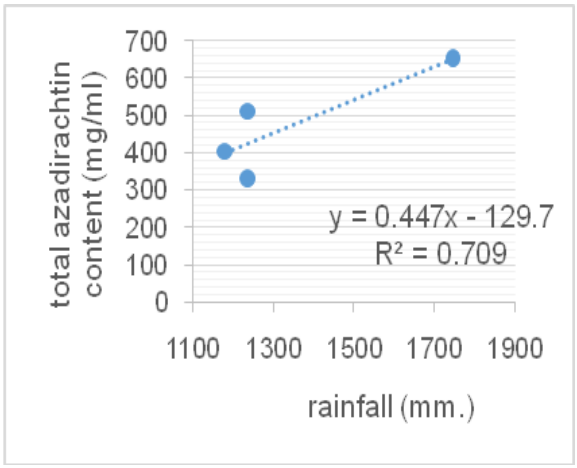
Result and discussion

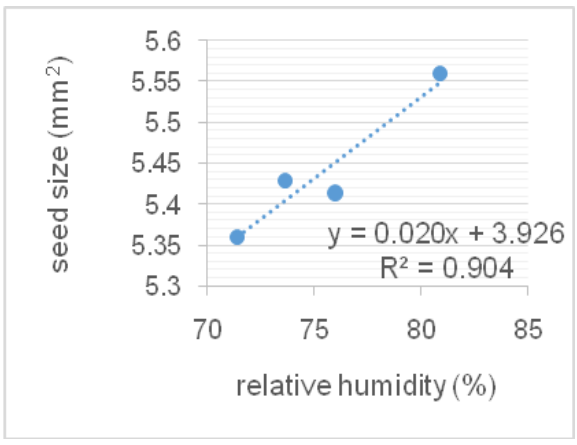
The neem fruits were collected from 34 provinces of Thailand covering locations in latitude 6.6717 - 20.0091, longitude 99.0038 - 104.8480, altitude 1 - 467 meters above sea level, with the climatic conditions of temperature (11.4 - 41.9 °C), humidity (73 – 80 %), annual rain fall (1,065.0 - 2,251.8 mm.) and the soil pH (3.0 - 8.5).

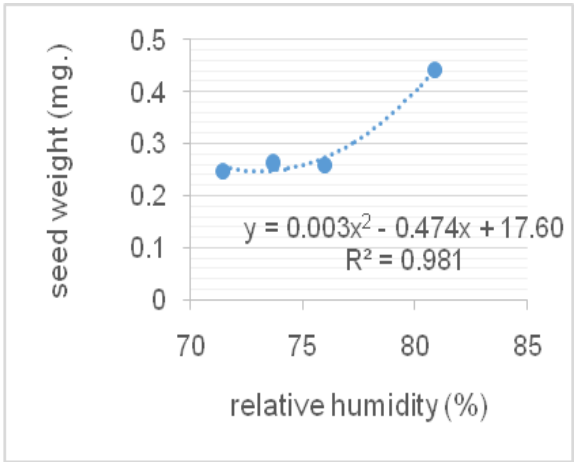


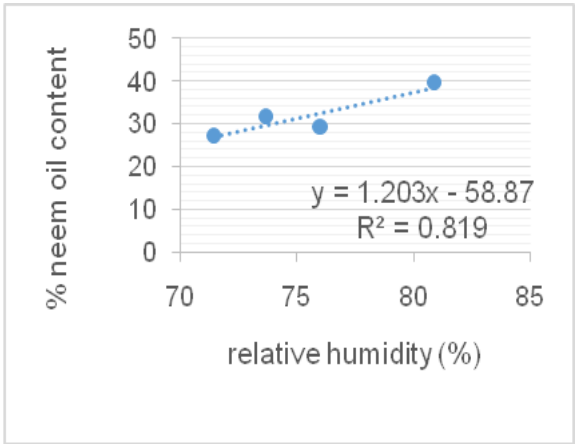


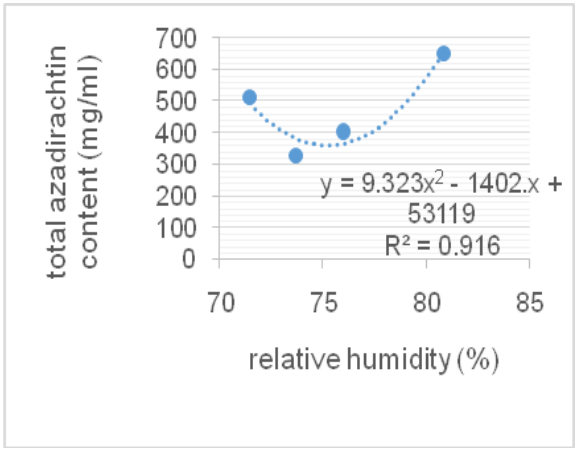


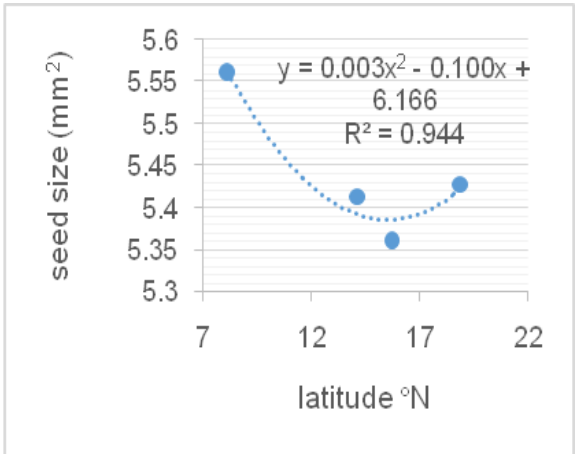


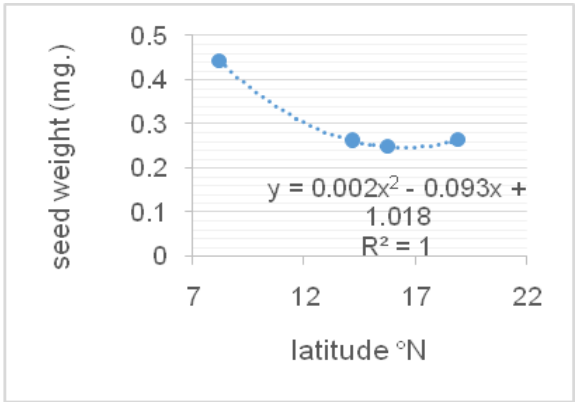


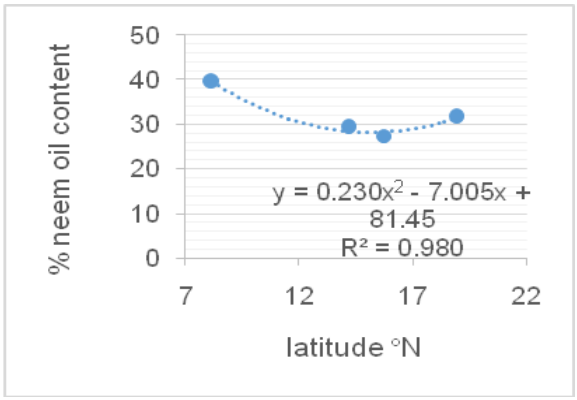


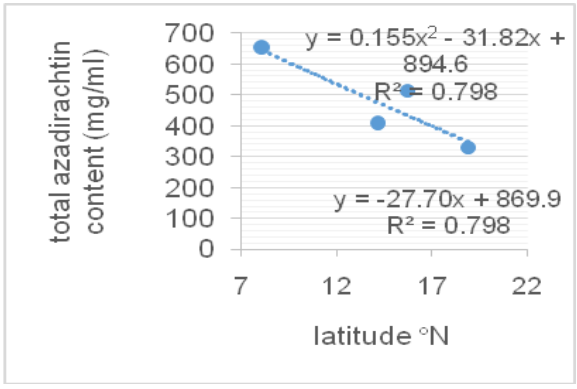












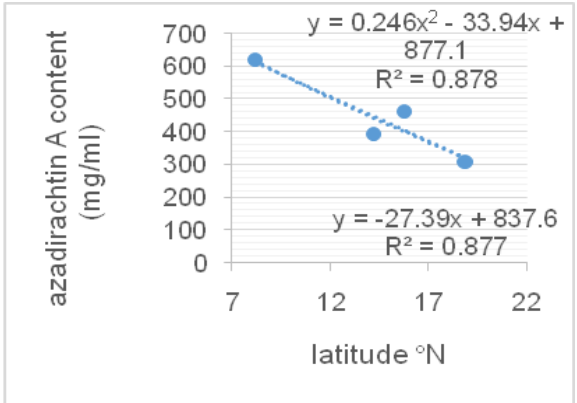


Fig. 2. Regression analyses between environmental factors: annual rainfall (mm), relative humidity (%) and Latitude (°N) and neem seed size (mm²), neem seed weight (mg.), neem oil content (%), total Aza content extracted from neem seed (mg/ml) collected from 4 climatic zones of Thailand.

The color of ripened neem fruits collected was yellowish green and only some was yellow. The significant variation of average values of all seed parameters was observed among the 34 provinces. The seed weight ranged from 0.14 to 0.51 gm/seed and the neem seed size (length x width) ranged from 5.30 to 5.77 mm^2. The seed weight was poorly positively correlated to the seed sizes at 44.6 % (r = 0.446). The maximum value of seed weight was obtained from Pattani province (0.51 gm/seed) and that of seed size was from Phatthalung province (5.77 $mm.^2$). (Table 1).

Table 1. The average of weight, size of neem seed, neem oil content, Azadirachtin (Aza A, Aza B and total Aza) content of neem seed collected from 6 regions of Thailand.

Regions	Provinces	weight (gm/seed)	size (mm^2)	neem oil (%)	Aza A (mg/ml)	Aza B (mg/ml)	Total Aza (mg/ml)
Rain forest	Chiang Mai	0.25	5.41	37.24	498.94	0.00	498.94
	Chiang Rai	0.31	5.42	48.98	290.73	0.00	290.73
	Phayao	0.27	5.42	20.99	158.83	0.00	158.83
	Uttaradit	0.23	5.46	19.54	288.95	79.45	368.4
Hotsub - humid	Chai Nat	0.22	5.36	44.72	210.17	0.00	210.17
	Sing Buri	0.28	5.41	23.35	378.24	0.00	378.24
	Ayutthaya	0.24	5.38	31.08	89.15	0.00	89.15
	Lop Buri	0.34	5.42	36.54	349.47	0.00	349.47
	NakhonSawan	0.31	5.38	32.69	417.9	0.00	417.90
	KamphaengPhet	0.38	5.49	28.49	139.97	0.00	139.97
	Chachoengsao	0.35	5.45	24.08	1420.31	57.90	1478.21
	Chon Buri	0.27	5.37	31.27	433.95	0.00	433.95
	Rayong	0.28	5.46	15.52	297.49	0.00	297.49
	Sa Kaeo	0.28	5.40	38.13	422.32	0.00	422.32
	Kanchanaburi	0.18	5.51	30.53	152.20	0.00	152.20
	NakhonPathom	0.29	5.41	20.39	365.63	129.60	495.23
	Ratchaburi	0.16	5.35	39.71	699.97	26.60	726.57
	SuphanBuri	0.31	5.38	17.76	624.62	0.00	624.62
	UthaiThani	0.24	5.41	53.36	189.27	0.00	189.27
	Bangkok	0.34	5.40	28.91	340.30	0.00	340.30
	SamutSakhon	0.26	5.30	24.48	494.91	0.00	494.91
	SamutSongkhram	0.18	5.44	22.74	160.30	0.00	160.30
	Phetchaburi	0.14	5.47	20.77	416.57	0.00	416.57
	PrachuapKhiri Khan	0.17	5.49	21.75	290.55	0.00	290.55
Hotsemi -arid	KhonKaen	0.18	5.40	26.22	995.58	82.72	1078.3
	NakhonRatchasima	0.31	5.39	24.05	156.20	0.00	156.20
	UbonRatchathani	0.28	5.35	20.53	129.94	0.00	129.94
	MahaSarakham	0.29	5.30	34.38	424.04	111.53	535.57
	Mukdahan	0.19	5.34	31.52	612.92	56.37	669.29
Tropical climate	SuratThani	0.37	5.43	30.12	263.45	0.00	263.45
	Phatthalung	0.43	5.77	30.20	450.32	0.00	450.32
	Chumphon	0.44	5.60	39.51	251.35	0.00	251.35
	Songkhla	0.45	5.30	40.60	262.36	0.00	262.36
	Pattani	0.51	5.71	57.24	1876.35	152.28	2028.63

Neem oil content ranged from 15.52 to 57.24 % which was a normal level as found in the report of Sathya and Manivannan (2013) that the obtained oil yields from *A. indica* seed were 25 - 45 % on dry matter basis and Satyanandam *et al.* (2011) that the concentration of oil contained around 15 - 45 %. The highest percent neem oil in seeds was recorded from Pattani province (57.24 %) followed by UthaiThani (53.36 %) and Chiang Rai (48.98 %).

Table 2. Correlation between seed parameters and Aza A, Aza B and total Aza content extracted from neem seed (mg/ml) collected from 34 provinces of Thailand.

	size (mm^2)	weight (gm)	neem oil (%)	Aza A (mg/ml)	Aza B (mg/ml)	Total Aza (mg/ml)
size (mm^2)	1	0.446	0.152	0.308	0.152	0.302
weight (gm)		1	0.331	0.275	0.143	0.271
neem oil (%)			1	0.251	0.119	0.246
Aza A (mg/ml)				1	0.634	0.997
Aza B (mg/ml)					1	0.696
Total Aza (mg/ml)						1

The samples were analyzed for the total azadirachtin (total Aza), azadirachtin A (Aza A) and azadirachtin B (Aza B) content in seeds. The results revealed that the total Aza also had highly significant variation among provinces ranging from 89.15 to 2,028.63 mg/ml. The maximum amount being over 1,000 mg/ml of total Aza was recorded from Pattani, Chachoengsao and KhonKaen at 2,028.63, 1,478.21 and 1,078.3 mg/g., respectively. Aza A was the main component found in all locations ranged from 89.15 to 1,876.35 mg/ml, but Aza B was found only in 8 provinces: Uttaradit, KhonKaen, MahaSarakham, Mukdahan, Chachoengsao, NakhonPathom, Ratchaburi and Pattani ranged from 26.60 to 152.28 mg/ml.

Table 3. Variation of seed characters of neem seeds among 4 different climatic zones of Thailand.

Climatic zone	Phenotypic characteristics					
	size (mm^2)	weight (gm)	neem oil (%)	Aza A (mg/ml)	Aza B (mg/ml)	Total Aza(mg/ml)
rainforest	5.43^b	0.265^b	31.69^a	309.36^a	19.86^a	329.22^a
hot sub -humid	5.41^b	0.261^b	29.31^a	394.66^a	10.70^a	405.37^a
hot sem i-arid	5.36^b	0.249^b	27.34^a	463.74^a	50.12^a	513.86^a
tropical climate	5.56^a	0.443^a	39.54^a	620.77^a	30.45^a	651.22^a

Remark: Mean values within column followed by the superscript with the same letters are not significantly different according to DMRT test ($P< 0.05$).

The highest amount of Aza A and Aza B were recorded from Pattani province (1,876.35 and 152.28 mg/ml).

This variation of azadirachtin content and components in neem seeds among different origins was confirmed by the studies of Ermel *et al.* (1995), Rengasamy *et al.* (1993), Kumar and Parmar (1996) and Kaushik *et al.* (2007). The isomer Aza A is the most important component present in the Neem seed extract (Naves *et al.*, 2003).

The seed weight as well as seed size was positively correlated with the percent neem oil at 33.1 % (r = 0.331) and 15.2 % (r = 0.152). The Aza A, Aza B and total Aza content was poorly correlated with the percent neem oil at 25.1, 11.9 and 24.6 % (r = 0.251, 0.119 and 0.246) (Table 2). This result was similar to

reports by Kundu (1999), Ermel *et al.* (1984) and Tewari (1992) who found that morphological characteristics of seeds has a low correlation with oil production and azadirachtin, seed width and average weight of seeds. Moreover, Tewari *et al.* (2007) reported that the total seed yield was significantly negative with oil content and azadirachtin contents but did not occur in this study. Therefore, it was suggested that the large size and the heavy weight of seed could mean that it has potential of having higher amount of oil and azadirachtin content.

Thereafter, all provinces were arranged into 4 groups based on the climatic variation: rain forest, hot sub - humid, hot semi - arid and tropical climate zone and compared with all characters (neem size, neem weight, neem oil content and azadirachtin component), the variation of those were found as the same as a report from Kaushik *et al.* (2007) that the variation in azadirachtin content in the seeds of neem collected from twelve different states of India was affected by habitat and climate. The highest values of neem size, neem weight, neem oil, Aza A, and total Aza content were recorded from the tropical climate zone and that of Aza B was from hot semi-arid zone. However, the significant differences were found only on the neem size and weight (Table 3).

Among the environmental factors: temperature, annual rainfall, relative humidity, altitude, latitude and longitude, it is only annual rainfall, relative humidity and latitude that affected neem seed size and weight, neem oil and Azadirachtin content. When the amount of annual rainfall (mm.) in the respective zones increased from 700 – 3,200 mm a year, the neem seed size and weight, neem oil and total Aza content also increased at the rate of 0.0003, 0.0003, 0.0191 and 0.4473 folds, theoretically, based on the regression equation of $Y = 0.003x + 5.0392$ ($R^2 = 0.849$), $Y = 0.003x + 0.1648$ ($R^2 = 0.980$), $Y = 0.0191x + 6.18$ ($R^2 = 0.883$) and $Y = 0.447x – 129.76$ ($R^2 = 0.710$). Furthermore, the relative humidity showed the linear regression on the neem seed size, neem seed weight, neem oil and total Aza content based on the regression equation of $Y = 0.02x + 3.93$ ($R^2 = 0.900$), $Y = 0.02x - 0.161.27$ ($R^2 = 0.828$) and $Y = 1.20x – 58.87$ ($R^2 = 0.819$), respectively. Meanwhile, its influence on the total Aza was a polynomial response based on the equation of $Y = 9.32x^2 - 1402.7x + 53119$ ($R^2 = 0.917$) (Fig. 3). This result was similar to the report from Elteraifi (2004) which demonstrated that the oil and azadirachtin content of neem seeds collected from different eco zones of the Sudan increased with the increase in rainfall and Singh (1987) who reported that rainfall had a significant positive effect on the active ingredient. This result is in agreement with the findings of Baumgart (1991), where the areas with a high rainfall and Aza values tended to increase. Elteraifi *et al.* (2011) found that the area with high rainfall and high humidity has a tendency of high oil content and azadirachtin. Kumar and Parmar (1997) also investigated azadirachtin content from some neem ecotypes in India and reported the causes for variability in azadirachtin content as high rainfall, extreme drought, and high humidity. Elteraifi *et al.* (2011) suggested that the optimum rainfall was found to be 717.9 mm. However, in their work, the mean annual rainfall in all the respective climatic zones are 1,065 – 2,258 mm higher than 717.9 and oil and azadirachtin has tendency of increasing in amount.

Altitude, latitude and longitude are indices pointing to the location on earth, and this could be used to indicate the influence of the environmental condition or habitat of the neem trees and their products. This result revealed that the average latitude in each zone had a highly negative effect on size, weight, neem oil, total Aza and Aza A in different ways. The effect of latitude on the neem seed size, neem seed weight and neem oil content had polynomial response based on the equation of $Y = 0.003x^2 - 0.10x + 6.17$ ($R^2 = 0.944$), $Y = 0.002x^2 - 0.093x + 1.02$ ($R^2 = 1.00$) and $Y = 0.23x^2 - 7.0x + 81.46$ ($R^2 = 91.7$). Meanwhile, those on total Aza and Aza A had linear response at the decline rate of 27.71 and 27.40 folds, theoretically, based on the regression equation of $Y = - 27.7x + 869.9$ ($R^2 = 0.798$) and $Y = -27.40x + 837.68$ ($R^2 = 0.878$), respectively. This result indicate hat the neem trees grown in the climatic zone located at the higher degree north of latitude tended to have smaller neem

seed size, lighter neem seed weight, lower amount of neem oil and azadirachtin content. On the contrary, Elteraifi *et al.* (2011) and Xing min *et al.* (2012) reported that the altitude and latitude had a low influence on the amount of azadirachtin and Rengasamy *et al.* (1993) reported that the climate at the moderate altitude of about 500 meters had the highest amount of azadirachtin.

In this study, the highest amount of azadirachtin above 1,000 mg/ml was found from the neem seed collected from the tropical climate zone in Pattani province (2,028.63 mg/ml), followed by hot sub - humid zone in Chachoengsao province (1,478.21 mg/ml) and hot semi - arid zone in Khon Kaen province (1,078.3 mg/ml).

The trees of this population can be mass produced and the plantation expanded to obtain seeds with higher azadirachtin content in the future.

Conclusion

The influence of growth locations in 34 provinces and 4 different climatic conditions of Thailand on neem seed morphology, neem oil and azadirachtin content in neem seeds was determined.

The weight and size of the collected neem seed ranged a little larger than in the previous reports. The largest size, highest amount of neem oil and azadirachtin was found in the seed from the south of Thailand, Pattani, which was classified as tropical climatic zone. The annual rainfall and relative humidity positively and significantly influenced the neem seed size, neem seed weight, neem oil and total Aza content, meanwhile, the latitude had negative effect on those. From this result, the neem seeds from Pattani has a high potential for the development of insecticide from neem in the future.

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