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Nodule Growth and Nitrogen Fixation of Selected Soybean Cultivars under Different Soil Water Regimes

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ABSTRACT

Sensitivity of N_2 fixation to soil-drying has been considered as a major limiting factor to improving soybean yield. Seven soybean cultivars derived from various parents—namely, Nakhon Sawan 1 (NS-1), SJ.4, Sukhothai 1 (ST-1), Sukhothai 2, (ST-2), Sukhothai 3 (ST-3), Chiang Mai 60 (CM-60) and Chakkrabandhu 1 (CK-1) were evaluated for their nodulation and growth and yield in the first experiment. They were derived from various parents and their maturity differed from 90 to 110 d. Cultivars NS-1, ST-2 and ST-3 were selected to examine their nodule growth and N_2 fixation capability under different water regimes in the second experiment. Nitrogenease activity (NA) of the nodulated roots of soybean increased with an increase in nodule dry matter (NDM) until 15 d after vegetative stage 4 (15 DAV_4; R_4) and then declined at 22 DAV_4 (R_5) under the well-watered regime. Under a water regime with progressive soil drying, the NA of the nodulated roots significantly decreased by 17.73% of the control from 7 DAV_4 (R_3). Among the soybean cultivars, NS-1 showed higher nodulation and N_2 fixation capacity under both water regimes. The NA of nodulated roots severely diminished by 87.10% of control at 12 DAV_4 (R_4) under progressive soil drying and did not recover well after rewatering; nodule growth was similarly affected. There was a greater and significant correlation between NDM and other observed physiological parameters at the R_4 stage. NDM is the most fundamental parameter for selecting better soybean cultivars for N_2 fixation under both water regimes. The soil plant analysis development (SPAD:M-502) reading showed a highly significant correlation (r=0.84) with NA and other physiological parameters. SPAD readings could be used as a rapid assessment technique for the identification of potential cultivars with greater N_2 fixation for both water regimes by measuring the accumulated total N in the shoot biomass at harvest.

Keywords: soybean, nitrogen fixation, nitrogenase, nodulations, SPAD, shoot N concentration

INTRODUCTION

Nitrogen fixation is more sensitive to water deficits than many other physiological processes (Serraj *et al.,* 1999) and this may restrict the nitrogen supply and thus the yield in soybean, as under drought conditions, nitrogen fixation activity in nodules was depressed. Decreased nitrogenase activity is primarily caused by nodule dehydration and tissue damage (Huang *et al.,* 1975; Albrecht *et al.,* 1984). A decrease in the photo-assimilates was associated with reduced

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plant water status under drought conditions and subsequently affected N_2 fixation (Fellow *et al.,* 1984; Patterson and Hudak, 1996). Oxygen diffusion and sucrose substrate, which mediate N_2 fixation, were limited under water deficits (Cordon *et al.,* 1997; Gálevz *et al.,* 2005). In addition, both the limitation of carbon flux and nitrogen accumulation in nodules reduced N_2 fixation in soybean under drought stress (Laudera *et al.,* 2007). Nitrogenous compounds, ureides accumulation and the metabolism in leaves have also been implicated in the feedback inhibition of N_2 fixation under water limitations; therefore, it has been applied as an indicator for selecting soybean cultivars that are better at N2 fixation under drought conditions (Herridge, 1982; Sinclair *et al.,* 2003; King and Purcell, 2005). Much investigative effort has been spent on the response of N_2 fixation under drought conditions but few studies (Serraj and Sinclair, 1998) have reported on cultivar variation in nodule formation and growth under drought and there is little known about nodule growth after recovery from such progressive soil drying. Since genotypic differences in N_2 fixation under drought conditions exist among soybean cultivars, soybean yield has been improved through the development of cultivars with greater N_2 fixation ability (Sinclair *et al.,* 2007) and through innovations in other management aspects (Hungria and Vargas, 2000). The current study aimed to evaluate some soybean cultivars for N_2 fixation by examination of the nodule growth and N_2 fixation activity under different water regimes, and the relationships between the observed N_2 fixation-related parameters and yield.

MATERIALS AND METHODS

Initial greenhouse evaluation

Seven soybean cultivars, widely grown in Thailand (Table 1) were evaluated for differences in nodulation and physiological responses under a well-watered regime and a regime involving rewatering from progressive soil drying in a previous experiment in the greenhouse (Tint *et al.,* 2011 in press). The cultivars were derived from different parents and varied in maturity from 90-110 d (Field Crops Research Institute, Thailand, 2005; Win *et al.,* 2009). The experiment was conducted at the Central Laboratory and Greenhouse Complex, Kamphaengsaen, Nakhon Pathom, Thailand (14°01′ N, 99°58′ E), during October 2008–January 2009. Garden soil enriched in nutrients was used to fill plastic pots (diameter 25 cm, depth 25 cm) to a weight of 3.5 kg. Seeds were inoculated with commercial peat-based rhizobium inoculum prior to sowing. Nine seeds at three seeding spots were sown in a pot; thinning was carried out 11 days after sowing (DAS) to maintain three plants in a pot which was the equivalent of 270,000 plants ha^{-1}.

Table 1 Soybean cultivars used for evaluation in the first experiment.

Soybean cultivar	Parentage	Physiological maturity (d)
Nakhon Sawan 1(NS 1)	Doteung × Santamaria	80–90
SJ.4	Acadian × Tainung	100–110
Sukhothai 1(ST 1)	Shih Shih × SRF 400	100–110
Sukhothai 2 (ST 2)	7016 × Sukhothai 1	100–110
Sukhothai 3 (ST 3)	Fort Lamy × CM 6o	90–100
Chiang Mai 60 (CM 60)	Willams × SJ.4	90–100
Ckakkrabandhu 1	UFC 1 × Santa Rosa	110–120

Source: Field Crops Research Institute, Thailand (2005) and Win *et al.* (2009)

The nodule growth and development of the tested cultivars were examined under treatments of well-watered (control) and 15-days of withholding water (stress). A greenhouse experiment was arranged in a randomized complete block design and seven cultivars were used with three replications. Water treatments were started at the V_3 growth stage (V_3). Pots were equally watered using a measuring beaker. The control and stress treatments were watered at three-day intervals, except for the stress treatment during the water withholding stress period. To avoid permanent wilting, a minimum amount of water (100 mL plant^{-1}) was added to the stress plants at 13 DAV_3. Re-randomization was carried out twice a week from emergence until imposing the treatments.

Data were collected at 0, 5, 10 and 15 days after the V_3 growth stage (DAV_3) for each cultivar and 7 d after rewatering (22 DAV_3) from both water regimes which approximated V_3 and the reproductive growth stages R_2, R_3, R_4 and R_5 (Fehr *et al.,* 1977), respectively. Nodulated root samples were obtained by breaking the soil clod and gently shaking to obtain intact roots. After washing with tap water on a 1 mm screen, samples were surface dried with blotting paper. Nodules were detached from the roots and immediately weighed on a digital balance to determine their fresh weight followed by oven drying for 72 h at 70 °C to determine their dry weight. The shoots from three plants in each pot were cut and sealed in plastic bags and kept in an icebox to avoid dehydration. Samples were collected during 12:00 to 13:00 hours. In the laboratory, moisturized blotting tissue was used to conduct surface cleaning and drying followed immediately by weighing on the digital balance to determine the fresh weight and then drying in an oven at 70 °C for 72 h after which the dry weight was recorded. Soil from the water-stress treatment was taken in 80 cc samples at each sampling time during the dried down period to monitor the soil moisture status. The soil moisture percentage was calculated from the wet and dry weight of the soil sample. Plants were harvested at their physiological maturity (R_7).

Greenhouse verification for N_2 fixation

Three of the seven cultivars from the first experiment—NS-1, ST-2 and ST-3—were selected. All selected cultivars had semi-determinate growth type. The second experiment was conducted during April–July 2009 at the same location to determine the N_2 fixation capacity and nodulation. Soil from the first experiment was recycled. Soil was used to fill pots to a uniform weight of 3.75 kg. Pots were washed by flash flooding several times to leach out residual NO_3^-. Seeds were inoculated with commercial peat-based rhizobium and 15 seeds were sown at five seeding spots; thinning at 13 DAS left three plants per pot. The experiment was arranged in a randomized complete block. The treatments were started at V_4 (before first flower; R_1). Watering used the same regime as in the first experiment. Data were collected at 0, 7, 12 and 15 days after the V_4 growth stage (DAV_4) of each cultivar and 7 d after rewatering (22 DAV_4) which approximated V_4 and the reproductive growth stages R_2, R_3, R_4 and R_5 (Fehr *et al.,* 1977), respectively.

The mean of five SPAD readings (Model M-502) were taken around the mid-rib on the center of the second most fully expanded leaf and were used to determine the relative water content of the leaf. Leaf samples were collected during 12:00–13:00 hours and put in zip plastic bags and kept in the icebox to avoid dehydration during sampling. Nodulated root samples were obtained and added to 750 cc glass bottles. Seventy-five ml of acetylene gas was injected to obtain a gas-air mixture (1:9) and incubation was carried out in the laboratory under sufficient fluorescent lighting at a controlled temperature of (25 ± 1 °C) for 30 min. Gas samples (10 cc) were taken with a syringe and saved into vacuettes (vacuum tubes).

The tip of each tube was sealed with para-film. The tubes were kept in the refrigerator and subjected to gas chromatometry (HEWETT 5890 series II) equipped with a flame ionization detector to measured the ethylene concentration in the gas sample. After incubation with the gas mixture, the root samples were placed on a 1 mm screen, washed with tap water and surface cleaned with blotting paper. Cutting of the shoots, collection of the nodulated roots and incubation were carried out to obtain replicates during the period from 13:00–17:30 hours to maintain the freshness of the samples. Although nitrogenase activity in nodules declines slowly following the removal of the shoot from nodulated roots, the immediate assay of the nodulated roots over a short period and replication of the plant samples allows for meaningful analysis of treatment effects (Streeter, 2003). Nodules were detached and their number recorded followed by immediate weighing on the digital balance. Nodule samples were dried in an oven at 70 ° C for 72 h. The shoot and soil samples were also collected using the procedure adopted in the first experiment. Dried shoot samples were ground to pass through a 2-mm sieve and a subsample was ground to pass through a 1-mm sieve. Total N in the shoots was determined by the micro-Kjeldahl method for samples taken at 7, 12, 15 and 22 DAV_4. The accumulated total N in the shoot biomass (micrograms of N per gram of shoot dry matter) was calculated by multiplying the shoot dry weight by the shoot N concentration. The nitrogen and shoot biomass accumulation rates were determined by dividing the total changes in N and biomass between the two harvests. The nitrogen concentration in the accumulated biomass was determined by dividing the N accumulation rate by the biomass accumulation rate (King and Purcell, 2006) that was assumed to have been derived from N_2 fixation because the soil had been leached of mineral nutrients by flash flooding several times. Plants were harvested at their physiological maturity (R_7) for their seed yield. Statistical significances were tested at the 0.05 level and highly significant testing was at the 0.01 level.

RESULTS AND DISCUSSION

In the first experiment, the control plants maintained soil moisture of (58.3 ± 2.8)% throughout the observed period while the mean soil moisture percentage was (37.9 ± 12.7), (19.5 ± 6.2) and (17.4 ± 1.3)% at 5, 10 and 15 DAV_3 under stress conditions, respectively. During the sampling period, average day and night temperatures were (28.8 ± 1.1) °C and (18.8 ± 2.2) °C and the day length was approximately 11.5 h. Cultivar NS-1 produced the highest nodule dry matter (NDM) under the control water regime and SJ.4 had the highest NDM under the soil-drying regime among the tested cultivars, but this was not significant differently from NS-1 (Table 2). Moreover, differences in the nodule growth patterns of the cultivars were observed. ST-2 had the highest NDM reduction ratio relative to the control with a value of 0.8 at the end of the soil-drying period, but its NDM was not significantly different from ST-3 under progressive soil drying. After rewatering, the nodule growth increased in almost all of the cultivars, with the exception of CM-60. Recovery of nodule growth also varied among cultivars; ST-1, SJ.4, CK-1 and NS-1 showed rapid recovery, while ST-2 and ST-3 slowly recovered nodule growth after rewatering. Nodule mass increased with plant age until the end of the observed period (R_5) under the well-watered (control) conditions and thereafter declined due to the allocation of phototosynthates favoring the demands of the growing seed (Gomes and Sodek, 1987). In contrast, nodule growth was suppressed and declined with increased soil drying (Table 2). There was no interaction effect between water treatment and cultivar; therefore, tested cultivars showed their genotypic differences under both water regimes. It was observed that NDM at

Table 2 Total nodule dry weight (mg plant^{-1}) of soybean cultivars (Sb) at 0, 5, 10 ,15 and 22 DAV_3 under well-watered (Control) and rewatered from 15 days progressive soil drying (Stress) water regimes (W) in the first growing season (October 2008–January 2009).

Water regime	Cultivar	Observed time				
		0 DAV_3	5 DAV_3	10 DAV_3	15 DAV_3	22 DAV_3
Control	NS-1	90.78^{ab}	129.00^{b}	165.22^{ab}	234.44^{ab}	341.33^{a}
	SJ-4	86.78^{ab}	133.44^{b}	177.22^{ab}	223.56^{ab}	292.33^{ab}
	ST-1	101.22^{ab}	138.44^{b}	159.89^{b}	252.56^{ab}	282.44^{ab}
	ST-2	67.33^{b}	99.22^{c}	134.56^{b}	187.11^{b}	251.33^{ab}
	ST-3	85.11^{b}	110.89^{b}	165.33^{ab}	277.67^{a}	273.67^{b}
	CM-60	110.22^{a}	134.44^{a}	188.33^{a}	270.00^{a}	320.56^{ab}
	CK-1	109.78^{a}	147.22^{a}	203.22^{a}	250.11^{ab}	286.44^{ab}
Mean of control		93.03	127.52	170.54	242.21	292.59
Stress	NS-1	88.78^{b}	116.44 ^{b}	177.44^{a}	171.44^{a}	206.67^{a}
	SJ-4	87.89^{b}	127.00^{ab}	184.89^{a}	167.44^{a}	211.56^{a}
	ST-1	86.67^{b}	116.00^{b}	144.89^{ab}	122.33^{b}	161.56^{ab}
	ST-2	66.33^{b}	100.89^{b}	126.56^{b}	153.00^{ab}	156.56^{ab}
	ST-3	85.00^{b}	112.00^{b}	147.67^{ab}	153.78^{ab}	160.56^{ab}
	CM-60	120.22^{a}	141.44^{a}	168.89^{ab}	160.11^{ab}	133.89^{b}
	CK-1	119.22^{b}	150.22^{a}	169.44^{ab}	134.11^{b}	163.44^{ab}
Mean of stress		93.44	123.00	159.67	151.75	170.60
$LSD_{0.05}$ W		7.86	11.03	17.30	22.09	24.21
$LSD_{0.05}$ W × Sb		20.73	29.17	45.58	58.45	64.06
CV%		13.2	13.9	16.4	17.7	16.5

Means in a column followed by the same letter are not significantly different at $LSD_{0.05}$.

22 DAV_3 had a highly significant correlation with seed yield (r=0.84) and also with shoot dry matter (0.77) in the first experiment (Tint *et al.,* 2011).

In the second experiment, three of the seven cultivars from the first experiment—namely, NS-1, ST-2 and ST-3—were selected to evaluate their nodulation and N_2 fixation capacity under different soil water regimes. During the sampling period, the average day and night temperatures were (33.6 ± 2.1) and (24.1 ± 0.9) °C and the day length was approximately 12.8 h. Control plants maintained soil moisture of (50.4 ± 1.5)% throughout the observed period. Under water-stress conditions, the observed soil moisture percentages were (28.1.9 ± 3.9), (14.2 ± 0.8) and (13.3 ± 0.8)% at 7, 12, and 15 DAV_4, respectively. There were no significant differences among the cultivars in the remaining SM% at all observed times. The control plants maintained around (79.26 ± 3.7)% relative water content (RWC) in the leaves throughout the observed period while the decrease in the RWC of the leaves under water stress was highly significant with increased soil drying and was maintained at (51.6 ± 4.2) , (47.2 ± 6.1) and (43.3 ± 6.6)% at 7, 12 and 15 DAV_4, respectively. However, there were no significant differences in the RWC of the leaves of the tested cultivars at each observed time within the same water regime and no interaction between water regimes and cultivars.

Shoot dry matter (SDM) increased with crop age under the well-watered regime. Under the progressive soil-drying regime, the reduction in the SDM was highly significant from 12 DAV_4

(Table 3). There were no significant differences in the SDM among the cultivars under the stress water regime except at 7 DAV_4. The SDM of most cultivars still decreased after rewatering at 16 DAV_4 due to continued leaf senescence (Brevedan and Edli, 2003) and probably the utilization of reserved food for their new growth (Fellow *et al.*, 1984).

Root growth under water stress was greater than in the control at 12 DAV_4 but the difference was not significant. There were no significant differences among tested cultivars within the same water regime, and among interactions as well (data not shown). Nodule dry matter increased over time under the control water regime. By withholding water, nodule growth was suppressed and was highly significant from 7 DAV_4. Nodule growth was significantly decreased under drought stress conditions compared to the control. There were no interactions between cultivars and water regimes throughout the observed times. Genotypic differences were found only under the control regime in early observations and the final observation (Table 4). Both the total nodule number (TNN) and individual nodule dry weight (INDW) significantly declined at 12 DAV_4. An inverse relationship existed between TNN and INDW under both water regimes. NS-1 had the greatest NDW as a result of having the highest TNN in both growing seasons compared with the same three cultivars. Sinclair *et al.* (1991) reported genotypic variations in NDW and TNN that were consistent across years and locations. Fellow *et al.* (1984) reported that nodule activity fully recovered from the withholding water stress imposed for 7 d at the V_7 stage within 2 d of rewatering. However, in the current study, the NDM of all cultivars did not recover well after rewatering, probably due to longer dry period and reduced allocation of photo-assimilates to the nodules when plants were in seed-filling stage (Gomes and Sodek, 1987).

As a result of using the recycled soil, the several soil washings, the high seeding rate and a slight delay in thinning, all cultivars showed temporary nitrogen deficiency symptoms with early yellowing in the cotyledons and leaf chlorosis around the V_3 stage, but this disappeared later. Therefore, it was assumed that plants received nitrogen mainly from N_2 fixation in this experiment.

Table 3 Shoot dry matter accumulation (g plant $^{-1}$) of soybean cultivars (Sb) at 0, 7, 12, 15 and 22 DAV_4 under well-watered (Control) and rewatered from 15 days progressive soil drying (Stress) water regimes (W).

Water regime	Cultivar	Observed time				
		0 DAV_4	7 DAV_4	12 DAV_4	15 DAV_4	22DAV_4
Control	NS-1	1.02^{a}	2.13 ^{a}	2.76 ^{a}	3.85ab	6.32
	ST-2	0.75^{b}	1.82^{ab}	3.25^{ab}	4.52a	6.75
	ST-3	0.62^{b}	1.55 ^{b}	2.59 ^{b}	3.50b	5.16
Mean of control		0.79	1.83	2.86	3.96	6.07
Stress	NS-1	0.80^{a}	2.08 ^{a}	2.33 ^{a}	2.38	1.65
	ST-2	0.83^{b}	1.75^{ab}	2.22 ^{a}	2.41	1.69
	ST-3	0.60^{b}	1.32 ^{b}	1.84 ^{a}	2.01	1.77
Mean of stress		0.80	1.71	2.13	2.27	1.70
$LSD_{0.05}$(W)		0.11	0.29	0.30	0.43	0.55
$LSD_{0.05}$(W × Sb)		0.19	0.50	0.52	0.74	0.95
CV%		12.8	15.4	11.4	13.1	13.5

Means in a column followed by the same letter are not significantly different at $LSD_{0.05}$.

Table 4 Total nodule number (plant $^{-1}$), total nodule dry weight (mg plant^{-1}) and individual nodule dry weight (mg nodule^{-1}) of soybean cultivars (Sb) at 0, 7, 12 ,15 and 22 DAV_4 under well-watered (Control) and rewatered from 15 days progressive soil drying (Stress) water regimes (W).

Water regime	Cultivar	Observed time				
		0 DAV_4	7 DAV_4	12 DAV_4	15 DAV_4	22DAV_4
Total nodule dry weight (mg plant^{-1})						
Control	NS-1	393^{a}	435^{a}	464^{a}	519^{a}	629^{a}
	ST-2	358^{ab}	402^{b}	438^{a}	517^{a}	600^{ab}
	ST-3	353^{b}	388^{b}	439^{a}	481^{a}	586^{a}
Mean of control		368	408	447	506	605
Stress	NS-1	383^{a}	387^{a}	375^{a}	335^{a}	333^{a}
	ST-2	367^{a}	386^{a}	395^{a}	342^{a}	323^{a}
	ST-3	368^{a}	382^{a}	352^{a}	345^{a}	326^{a}
Mean of stress		372	385	374	341	327
$LSD_{0.05}$ W		11.25	15.13	29.15	22.79	15.37
$LSD_{0.05}$ W × Sb		19.5	26.2	50.5	39.5	26.6
CV%		2.9	3.6	6.8	5.1	3.1
Total number of nodules plant^{-1}						
Control	NS-1	52.78^{a}	62.11^{a}	65.22^{a}	71.22^{b}	93.56^{ab}
	ST-2	33.67^{ab}	51.67^{ab}	65.78^{a}	80.55^{a}	119.89^{a}
	ST-3	28.22^{b}	42.56^{b}	51.33^{a}	55.22^{c}	79.22^{b}
Mean of control		38.22	52.11	60.78	68.96	97.56
Stress	NS-1	56.78^{a}	45.79^{a}	43.15^{a}	23.78	27.11^{a}
	ST-2	33.11^{b}	42.78^{a}	30.11^{ab}	21.33^{a}	18.67^{a}
	ST-3	34.00^{b}	38.78^{a}	22.78^{b}	18.00^{a}	19.33^{a}
Mean of stress		41.30	42.11	32.01	21.04^{a}	21.70^{a}
$LSD_{0.05}$ W		9.24	10.39	10.25	5.43	21.43
$LSD_{0.05}$ W × Sb		16.00	18.00	17.76	9.40	37.12
CV%		22.10	21.0	21.0	11.5	34.2
Individual nodule dry weight (mg nodule^{-1})						
Control	NS-1	7.46^{a}	7.19^{a}	7.19^{a}	7.30^{a}	6.78^{a}
	ST-2	11.45^{ab}	7.92^{a}	6.80^{a}	6.46^{a}	5.39^{a}
	ST-3	12.80^{a}	9.19^{a}	8.58^{a}	8.79^{a}	7.49^{a}
Mean of control		9.67	8.10	7.52	7.52	6.55
Stress	NS-1	6.92^{b}	8.78^{a}	9.46^{b}	14.25^{b}	13.46^{a}
	ST-2	12.02^{a}	9.83^{a}	14.48^{ab}	16.67^{a}	23.32^{a}
	ST-3	10.99^{ab}	10.07^{a}	20.54^{a}	21.51^{a}	17.54^{a}
Mean of stress		10.88	9.56	14.83	17.25	18.1
$LSD_{0.05}$ W		2.40	2.11	4.45	3.59	7.49
$LSD_{0.05}$ W × Sb		4.17	3.66	7.71	6.23	12.88
CV%		22.3	22.8	37.9	27.4	57.4

Means in a column followed by the same letter are not significantly different at $LSD_{0.05}$.

A chlorophyll meter (SPAD:M-502) has been widely used to assess chlorophyll content. Significant correlations between photosynthesis and SPAD M-502 readings have been documented for a large number of species, including soybean (Ma *et al.,* 1995). However, Fritschi and Ray (2007) reported that although there was a highly significant correlation between the SPAD reading and the total chlorophyll content, the SPAD reading was not sufficient to predict the leaf N content across a large number of soybean germplasm. On the other hand, both the leaf chlorophyll content and the shoot total N were also positively correlated with the SPAD readings in soybean (Mirza *et al.,* 1990). The SPAD reading has been applied to differentiate between promiscuous and nonpromiscuous nodulation in soybean (Gwata *et al.,* 2004); they reported that the leaf color score transformed from the readings had a positive highly significant correlation with NDW. Therefore, SPAD was also used to examine its relationship with the observed parameters in the current study. The SPAD reading had significantly declined from12 DAV_4 under the soil dry water regime (15 DAV_4). There was no genotypic difference of SPAD reading within the same water regime (Table 5).

Nitrogen concentration in the accumulated biomass was used to compare the N_2 fixation ability of the soybean cultivars under both water regimes (Sinclair *et al.,* 2000; King and Purcell, 2006). They suggested that the shoot N concentration of well-watered plants is more likely to be a better indicator of the genotypic response of shoot N concentration to drought stress than the shoot ureides of well-watered plants. In the current study, there was highly significant decline in both the nitrogen and shoot biomass accumulation rates under the stress regime as compared with the control regime (Table 6). The N concentration in the accumulated shoot biomass in the control plants was higher than in the stressed plants, which was a similar result to that reported by King and Purcell (2006). In addition, the results were consistent with the higher SPAD reading for the leaves of control plants (Table 5). There were no interactions between cultivar and water stress in the accumulation rates and the N concentration in the accumulated biomass.

Nitrogen concentration in the shoot mass

Table 5 SPAD readings of the second most fully expanded leaf of soybean cultivars (Sb) at 0, 7, 12 and 15 DAV_4 under well-watered (Control) and rewatered from 15 days progressive soil drying (Stress) water regimes (W).

Water regime	Cultivar	Observed time				
		0 DAV_4	7 DAV_4	12 DAV_4	15 DAV_4	22DAV_4
Control	NS-1	29.8^a	33.7^a	36.1^a	36.8^a	36.4^a
	ST-2	28.5^a	30.0^a	35.1^a	36.9^a	35.2^a
	ST-3	30.6^a	33.8^a	35.6^a	37.5^a	40.3^a
Mean of control		29.6	32.5	35.6	37.1	36.7
Stress	NS-1	30.7^a	32.6^a	32.5^a	28. 4^a	37.3^a
	ST-2	28.7^a	29.8^a	31.0^a	27.1^a	34.7^a
	ST-3	29.3^a	32.7^b	34.3^a	29.8^a	36.7^a
Mean of stress		29.6	31.7	32.6	28.7	36.2
$LSD_{0.05}$(W)		0.13	0.21	0.26	0.32	0.34
$LSD_{0.05}$(W × Sb)		2.3	3.7	4.5	5.6	5.9
CV%		4.2	6.3	7.3	9.4	8.9

Means in a column followed by the same letter are not significantly different at $LSD_{0.05}$.

could be used to estimate the N_2 fixation capacity and this would allow the assessment of many samples. However, the acetylene reduction assay (ARA) provides a more precise estimate of the N_2 fixation ability and is widely accepted, because acetylene strongly interacts with all forms of substrates for the nitrogenase enzyme (Hardy *et al.,* 1968). In the current study, nitrogenase activities (NA) of the selected cultivars under both water regimes were also evaluated by ARA (Table 7).

Under the control regime, the NA of nodulated roots in all cultivars showed a gradual increase with increasing nodule mass until 15

Table 6 Accumulation rates of nitrogen and biomass, nitrogen concentration in accumulated shoot biomass between 7 and 15 DAV_4 of soybean cultivars (Sb) under well-watered (Control) and progressive soil drying (Stress) water regimes (W).

Water regime	Cultivar	Accumulation rate N (μg plant^{-1} d^{-1})	Accumulation rate Shoot biomass (g plant^{-1} d^{-1})	N concentration in accumulated shoot mass (μg N g^{-1}shoot mass)
Control	NS-1	3.10^a	0.21^b	14.24^a
	ST-2	3.78^a	0.34^a	11.31^a
	ST-3	3.37^a	0.24^b	13.98^a
Mean of control		3.42	0.266	13.17
Stress	NS-1	0.53^a	0.04^b	14.00^a
	ST-2	0.54^a	0.08^a	7.33^a
	ST-3	0.76^a	0.09^a	9.23^a
Mean of stress		0.61	0.07	10.18
$LSD_{0.05}$ W		0.66	0.05	4.58
$LSD_{0.05}$ W × Sb		1.14	0.08	7.92
CV%		31.0	28.0	37.3

Means in a column followed by the same letter are not significantly different at $LSD_{0.05}$.

Table 7 ARA (μmol C_2H_4 hr^{-1} plant^{-1}) of fresh nodule and root mass of soybean cultivars (Sb) at 7, 12, 15 and 22DAV_4 under well watered (Control) and rewatered from 15 days progressive soil drying (Stress) water regimes (W).

Cultivar	7 DAV_4		12DAV_4		15DAV_4		22DAV_4	
	Control	Stress	Control	Stress	Control	Stress	Control	Stress
NS-1	19.96^a	15.27^a	24.28^a	2.80^a	27.13^b	1.94^a	14.44^c	5.46^a
ST-2	15.24^b	13.69^{ab}	16.15^{ab}	2.10^a	31.49^{ab}	2.10^a	26.58^a	4.67^a
ST-3	14.21^b	12.67^b	13.30^b	2.06^a	37.87^a	2.37^a	22.85^b	4.76^a
Mean	16.47	13.88	17.91	2.31^a	32.17	2.14	21.29	4.96
$LSD_{0.05}$ W	2.14		1.78		3.17		3.02	
$LSD_{0.05}$ W × Sb	3.71		3.08		5.49		5.23	
CV%	13.5		16.7		17.6		21.9	
Relative NA (%)								
NS-1	76.50		11.53		7.15		37.81	
ST-2	89.83		13.00		6.67		17.57	
ST-3	84.27		12.89		6.65		20.83	

Means in a column followed by the same letter are not significantly different at $LSD_{0.05}$.

DAV_4. In contrast, the ARA results declined at 22 DAV_4 (R_5 stage). This indicated there was limitation in the amount of substrate from the photo-assimilates available to nodules which caused a decline in NA under the well-watered regime (Gomes and Sodek, 1987) in a similar manner to that reported for NDW. Under the control regime, NS-1 showed the highest NA in early periods, but ST-3 was the highest during the later period because NS-1 was a relatively early maturing cultivar.

Like nodule growth, nitrogenase activity was also depressed under the stressed regime. NA under the stressed regime significantly decreased from 7 DAV_4 while plants maintained RWC in the leaves of around (53.07 ± 5.3)% whereas soil moisture remained at (28.1.9 ± 3.9)% . However, at 7 DAV_4, the SDM had not significantly decreased under the progressive drying regime (Table 3). This fact, in conjunction with the results for SDM and NA, indicated that N_2 fixation was affected much earlier than shoot growth. Steeter (2003) reported that the major decrease in N_2 fixation activity was due to the lower demand for fixed N to support growth, not due to carbon supply to bacteriods. On the other hand, a limitation of carbon supply to nodules involved in the decline of N_2 fixation under early droughting has been reported (Gálevz *et al*., 2005; Laudera *et al.,* 2007). Huang *et al*. (1975) also reported that the inhibition of shoot photosynthesis accounted for inhibition in N_2 fixation in soybean at low water potential. According to Wang *et al*. (2006), soybean net photosynthesis dramatically declined when the remaining soil moisture reached 26% which was close to the level at 7 DAV_4 in the current study. Accumulated evidence suggested that the decline in N_2 fixation was associated with a decline in photosynthesis under drought conditions; therefore, the two processes were interdependent. In addition, dehydration and tissue damage in nodules (Huang *et al.,* 1975; Albrecht *et al.,* 1984) low O_2 diffusion (Cordon *et al.,* 1997), a dramatic decrease in sucrose synthase activities in nodules (González *et al.,* 1995), limitations of carbon flux and sucrose (Gálevz *et al.,* 2005), and feedback inhibition of N accumulation in leaves and nodule (Laudera *et al.,* 2007), have all been reported concerning the sensitivity of NA to moisture stress.

NA severely declined about eightfold at 12 DAV_4 while plants maintained RWC in the leaves at around (48.50 ± 7.8)% and the soil moisture remained at (14.2 ± 0.8)%. There was no significant difference among the cultivars at 12 DAV_4 under the progressive drying regime. NS-1 had the highest NA under both water regimes

The relative NA of nodulated roots from stressed plants varied with the crop development stage and nodule development along with the intensity of stress (Table 7). No interaction between water treatments and cultivars was found at 7 DAV_4, but did appear in later observations due to ARA differences among cultivars under the well-watered regime. At the end of the soil-drying period, NS-1 sustained greater NA (7.15%) than ST-2 (6.67%) and ST-3 (6.65%), which was similar to their rankings for N concentration in the accumulated shoot biomass and the tolerance ratio. With regard to the results of N concentration in the accumulated shoot biomass (Table 6) and NA under both regimes (Table 7), NS-1 was more likely to have higher N_2 fixation under both regimes.

Relationships between NDM and other observed physiological parameters varied with the observed times. The highest significant correlations were found at 15 DAV_4 (R_4 stage) as presented in Table 8. NDW had highly significant correlation coefficient values with the observed physiological parameters at R_4, and finally with seed yield as tested under the different water regimes. The SPAD readings also showed good significant correlations with the observed parameters. However, it is likely to be more meaningful to identify the N_2 fixation potential of

Table 8 Simple correlation between specific leaf weight (SLW) and other observed parameters at 15 DAV_4 (R_4 stage).

	NDM	NA	SDM	RDM	SPAD	AcN	$Nconc_{7-15}$	SdY
NDM	1	0.91^{**}	0.87^{**}	0.62^{**}	0.85^{**}	0.91^{**}	0.30^{ns}	0.65^{**}
NA		1	0.84^{**}	0.64^{**}	0.84^{**}	0.88^{**}	0.34^{ns}	0.79^{**}
SDM			1	0.64^{**}	0.78^{**}	0.97^{**}	0.20^{ns}	0.55^{*}
RDM				1	0.59^{**}	0.67^{**}	0.29^{ns}	0.24^{ns}
SPAD					1	0.82^{**}	0.32^{ns}	0.73^{**}
AcN						1	0.33^{ns}	0.61^{**}
$Nconc_{7-15}$							1	0.29^{ns}
SdY								1

NDM = Nodule dry weight (mg $plant^{-1}$), NA = Nitrogenease activity by ARA (μmol C_2H_4 hr^{-1} $plant^{-1}$); SDM = Shoot dry matter(g $plant^{-1}$); RDM = Root dry matter (g $plant^{-1}$); SPAD = SPAD reading; AcN = Accumulated total N in the shoot biomass at harvest (μg g^{-1} of SDM);

$Nconc_{7-15}$ = Concentration of total N in accumulate shoot biomass between 7 and 15 DAV_4 harvest (μg g^{-1} of SDM); SdY = Seed yield (g $plant^{-1}$); ns = Not significant; * = Significant at $P \leq 0.05$; ** = Significant at $P \leq 0.01$; n=18.

tolerant soybean cultivars by the amount of total N accumulated in the harvested shoot biomass at the time of interest, rather than by the SPAD reading alone as a quick assessment.

CONCLUSION

NA in the nodulated roots of soybean increased with increased nodule mass due to plant development until 15 DAV_4 (R_4) and then declined with a decrease in nodule growth at 22 DAV_4 (R_5) under the well-watered regime. Under the progressive drying regime, the NA of nodulated roots significantly decreased by 17.73% of control from 7 DAV_4 (R_3), while plants maintained RWC in the leaves at around (53.07 ± 5.3)% and the soil moisture remained at (28.1.9 ± 3.9)%. NA of nodulated roots was severely diminished by 87.10% of the control at 12 DAV_4 (R_4) while plants maintained RWC in the leaves at around (48.50 ± 7.8)% and the soil moisture remained at (14.2 ± 0.8)% and did not recover well after rewatering. The same was also true for nodule growth. Based on the results of relative ARA and N concentration accumulated in the shoots, the NS-1 cultivar among the tested cultivars was more likely to be tolerant to soil drying. A greater significant correlation between NDM and other observed physiological parameters was found at the R_4 stage. NDM is the most fundamental parameter in selecting for soybean cultivars with greater N_2 fixation ability under both water regimes. SPAD readings could be used as a rapid assessment technique for the identification of potential cultivars with greater N_2 fixation for both water regimes by measuring the accumulated total N in the shoot biomass at harvest, as mentioned above. Further research is needed to investigate whether the N_2 fixation tolerance characteristic of cultivars under water stress could lead to increase yields under drought stress among similar yielding genotypes without excessive soil drying and other nutritional effects.

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LITERATURE CITED

Albrecht, S.L., J.M. Bennett and K.J. Boote. 1984. Relationship of nitrogenase activity to plant water stress in field-grown soybeans. **Field Crops Res.** 8: 61–71.

Brevedan, R.E. and D.B. Egli. 2003. Short periods of water stress during seed filling, leaf senescence, and yield of soybean. **Crop Sci.** 43: 2083–2088.

Cordon A.J., F.R. Minchin, L. Sksφt and C.L. James. 1997. Stress-induced declines in soybean N_2 fixation are related to nodule sucrose synthase activity. **Plant Physiol.** 114: 937–946.

Fehr, W.R.C., C.E. Caviness, D.T. Burmood and J.S. Pennington. 1977. Stages of development descriptions of soybeans (*Glycine max* L. Merri1). **Crop Sci.** 11: 929–931.

Fellow, R.J., R.P. Patterson, C.D. Rapper, Jr. and D. Harris. 1984. Nodule activity and allocation of photosynthate of soybean during recovery from water stress. **Plant Physiol.** 84: 456–460.

Field Crops Research Institute, Thailand. 2005. **A Guide Book for Field Crops Production in Thailand.** Department of Agriculture. Ministry of Agriculture and Co-operative. Bangkok, Thailand. 152pp.

Fritschi, F.B. and J.D. Ray. 2007. Soybean leaf nitrogen, chlorophyll content, and chlorophyll *a*/*b* ratio. **Photosynthetica** 45(1): 92–98.

Gálevz, L., M.E. González and C. Arrese-Igor. 2005. Evidence for carbon flux shortage and strong carbon/nitrogen interactions in pea nodules at early stages of water stress. **J. Exp. Bot.** 56(419): 2551–2561.

Gomes, M.A.F and L. Sodek. 1987. Reproductive development and nitrogen fixation in soybean (*Glycine max* [L.] Merril.) **J. Exp. Bot.** 38(197): 1982–1987.

González, E.M., A.J. Gordon, C.L. James and C. Arrese-Igor. 1995. The role of sucrose synthase in the response of soybean nodules to drought. **J. Exp. Bot.** 46(10): 1515–1523.

Gwata, E.T., D.S. Wofford, K.J. Boote, A.R. Blount and P.L. Pfahler. 2004. Inheritance of promiscuous nodulation in soybean. **Crop Sci.** 45: 635–638.

Hardy, R.W.F., R.D. Holsten, E.K. Jackson and R.C. Burns. 1968. The acetylene-ethylene assay for N_2 fixation: Laboratory and field evaluation. **Plant Physiol.** 43: 1185–1207.

Herridge, D.F. 1982. Relative abundance of ureides and nitrate in plant tissue of soybean as a quantitative assay of nitrogen fixation. **Plant Physiol.** 70: 1–6.

Huang, C-Y., J.S. Boyer and L.N. Vanderhoef. 1975. Limitation of acetylene reduction (nitrogen fixation) by photosynthesis in soybean having low water potentials. **Plant Physiol.** 56: 228–232.

Hungria, M. and A.T. Vargas. 2000. Environmental factors affecting N_2 fixation in grain legumes in the tropics, with an emphasis on Brazil. **Field Crops Res.** 65: 151–164.

King, C.A. and L.C. Purcell, 2005. Inhibition of N_2 fixation in soybean is associated with elevated ureides and amino acids. **Plant Physiol.** 137: 1389–1396.

King, C.A. and L.C. Purcell. 2006. Genotypic variation for shoot N concentration and response to water deficits in soybean. **Crop Sci.** 46: 2396–2402.

Laudera, R., D. Marino, E. Larrainzar, E.M. González and C. Arrese-Igor. 2007. Reduced carbon availability to bacteroids and elevated ureides in nodules, but not in shoots, are involved in the nitrogen fixation response to early drought in soybean. **Plant Physiol.** 145: 539–546.

Ma, B.L., M.J. Morrison and H.D. Voldeng. 1995. Leaf greenness and photosynthetic rate in soybean. **Crop Sci.** 35: 1411–1414.

Mirza, N.A., B.B. Bohlool and P. Somasegaran. 1990. Nondestructive chlorophyll assay for *Bradirhizobium japonicum*. **Soil Biol. Biochem.** 22: 203–207.

Patterson, R.P. and C.M. Hudak. 1996. Drought-avoidant soybean germplasm maintains nitrogen-fixation capacity under water stress. **Plant and Soil** 186: 37–43.

Serraj, R. and T.R. Sinclair. 1998. Soybean cultivar variability for nodule formation and growth under drought. **Plant and Soil** 202: 159–166.

Serraj, R., T.R. Sinclair and L.C. Purcell. 1999. Symbiotic N_2 fixation response to drought. **J. Exp. Bot.** 50: 143–155.

Sinclair, T.R., A.R. Soffes, K. Hinson, S.L. Albrecht and P.L. Pfahler. 1991. Genotypic variation in soybean nodule number and weight. **Crop Sci.** 31 (2): 301–304.

Sinclair, T.R., L.C. Purcell, V. Vadez, R. Serraj, C.A. King and R. Nelson. 2000. Identification of soybean genotypes with N_2 fixation tolerance to water deficits. **Crop Sci.** 40: 1803–1809.

Sinclair, T.R., V. Vadez and K. Chen. 2003. Ureide accumlulation in response to Mn nutrition by eight-soybean genotypes with N2 fixation tolerance to soil drying. **Crop Sci.** 43: 592–597.

Sinclair, T.R., L.C. Purcell, C.A. King, C.H. Sneller, P. Chen and V. Vadez. 2007**.** Drought tolerance and yield increase of soybean resulting from improved symbiotic N_2 fixation. **Field Cr ops Res.** 101(20): 38–71.

Streeter, J.G. 2003. Effects of drought on nitrogen fixation in soybean root nodules. **Plant Cell Environ.** 2:1199–1204.

Tint, A.M.M., E. Sarobol, S. Nakasathein and W. Chai-aree. 2011. Differential responses of selected soybean cultivars to drought stress and their drought tolerant attributions. **Kasetsart J**. **(Nat. Sci.).** 45(4): 571–582.

Wang, L., Z. Tong and D. Shengyan. 2006. Effect of drought and rewatering on photosynthetic physioecological characteristics of soybean. **Acta Ecologica Sinica.** 26(7): 2073–2078.

Win, N.P.P., P. Sripichitt, W. Chanparaset, V. Hongtrakul and C. Phumichai. 2009. Evaluation of soybean [*Glycine max* (L.) Merrill] germplasm for field weathering resistance using seed quality and SCAR markers. **Kasetsart J**. **(Nat. Sci.).** 43: 629–641.