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Variety and Seeding Time Influence on Flowering Characteristics and Trichodesmine Content in Sunn Hemp (*Crotalaria juncea* L.)

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ABSTRACT

Background: Sunn hemp (*Crotalaria juncea* L.) is an important annual legume used as green manure. It contains pyrrolizidine alkaloids, which are apparently effective in suppressing nematodes. It is pertinent to know the growth stage during which pyrrolizidine alkaloid concentrations become higher.

Methods: The period of days necessary for flowering, pod growth and the concentrations of one pyrrolizidine alkaloid in pods at different stages in five sunn hemp cultivars with May and July seeding were investigated.

Result: Findings indicated that flowering of the two cultivars occurred later than the others, especially for May seeding. The pods grew to 20 mm length in 6-11 days from flowering. Then the pods grew to maximum size (40 mm length) in only few days. A main pyrrolizidine alkaloid in sunn hemp seed, trichodesmine, increases as pod size grow; then it increases considerably as pod mature. However, it in matured pod hull contains similar values of whole immature pod. These results demonstrate that both day-neutral and short-day cultivars with July seeding shorten the days to first flower and indicate that cutting after 10 days from first flowering is effective for increasing pyrrolizidine alkaloid concentration.

Key words: *Crotalaria juncea* L., Flowering characteristics, Green manure legume, Nematode suppression, Pod mature, Pyrrolizidine alkaloid.

INTRODUCTION

Sunn hemp, one of *Crotalaria* spp., used mainly in agriculture (Mosjidis, 2006), includes over 500 tropical and subtropical species (Polhill, 1981). It is used for many purposes, but particularly as green manure and fibre (Purseglove, 1968). It is also used for suppression of specific nematodes. As reviewed by Wang *et al*. (2002), sunn hemp suppresses sedentary endoparasitic nematodes. Although details of this suppression mechanism remain unclear, the secondary plant metabolites, pyrrolizidine alkaloids (PAs), are reportedly related to the nematicidal effect (Thoden and Boppré, 2010). The *Crotalaria* genus includes species containing PAs. Sunn hemp seeds include PAs, particularly trichodesmine and junceine (Ji *et al*. 2005; Smith and Culvenor, 1981). Mosjidis *et al*. (2012) reported that sunn hemp stems and leaves contain less PAs than seeds do. Nurhayati and Ober (2005) did not detect alkaloids from sunn hemp cotyledons, leaves, or flowers. The earlier study results indicating trichodesmine concentrations below 1 mg/kg in sunn hemp stems, leaves and flowers (Kaneko *et al*., 2021) support their results.

To increase PAs concentrations of sunn hemp green manure, two strategies are considered. One is using specific varieties. Sunn hemp has wide range of genetic divergence for agro-morphological characters (Maruthi *et al*., 2020), fibre yield (Maruthi *et al*., 2021) and flowering character (Desai *et al*., 2021). Flowering of sunn hemp depends on short-day and little or no seed is produced in the United States (White and Haun, 1965). Although the first sunn hemp commercialized cultivar in the United States 'Tropic Sun' is a short-day cultivar, a new cultivar from Alabama 'AU Golden' has a day-neutral characteristic (Meagher *et al*., 2017).

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Garzon *et al*. (2021) reported that two of five sunn hemp genotypes ('Blue Leaf' and 'Crescent Sunn') seeded in April in central Florida did not flower until late September, whereas 'Ubon', 'AU Golden' and 'Red mini' had flowered by August. These findings indicate that varieties of sunn hemp exist with different flowering characteristics. A comparison of 16 accessions by Cho *et al*. (2016) demonstrated that the days to first flower opening differed not only between accessions but also between planting seasons. Using day-neutral and early varieties, one can shorten the period until flowering or seed setting. Never the less, no report describes flowering characteristics of commercial sunn hemp cultivars for green manure in Japan under warm temperature conditions.

The second hypothesis is that if immature pods contain high PAs, it is better that they be cut after flowering but before pods have matured. From plant guides by the USDA NRCS (Sheahan, 2012), for use as green manure, sunn hemp is ploughed or disced before it reaches full-bloom stage and

thereafter becomes too fibrous for effective use. However, no report describes growth from the perspective of PAs concentrations during pod development of sunn hemp. This study investigated the number of days required for flowering at different seeding seasons and the period of pod growth and concentrations of one PA in pods with different stages of five sunn hemp cultivars. The findings clarify the time at which the PA concentration of the green manure is highest.

MATERIALS AND METHODS

Sunn hemp was cultivated at Kyushu Okinawa Agricultural Research Centre, NARO, Kumamoto, Japan (32°53′N, 130°44′E, 78 m a.s.l.) during May-October 2020. The data on average monthly temperature and precipitation are presented in Fig 1. Soil was heavy clay (Hydric Pachic Melanudandsby Soil Taxonomy 1999). The experimental field was cultivated by rotary tiller after buckwheat (*Fagopyrum esculentum* Moench) cultivation in 2019 summer. A compound fertilizer (N: P_2O_5: K_2O, 16%:16%:16%) was applied as a basal dose at rates equivalent to 30 kg N ha^{-1} immediately before sowing seeds. Commercial sunn hemp cultivars, 'Nekobukira (Takii and Co., Ltd)', 'Nemakorori '(Snow Brand Seed Co., Ltd.)','Kobutorisou (Sakata Seed Corp.)' and 'Kurotararia (Kaneko Seeds Co., Ltd.)' currently distributed in Japan for green manure with the nematicidal effect and one cultivar 'Ubon (Tropical seeds, LLC)' from USA were sown on 25 May and 21 July, 2020. The seeding rate was 60 kg ha^{-1}. A roller was used to increase seed-soil contact and level the field after sowing. The plot was 3 m × 10 m, distributed in a randomized complete block design with six replications for both seeding dates.

The day to first flowering and the period from flowering to podding of the three to five branches that had bloomed earliest for each plot were recorded. The period from flowering to podding for May seeding was not recorded because of the lack of pod formation for first flowering of the early varieties. Pods were harvested from each of three replications at May seeding plots of two cultivars, 'Nemakorori' and 'Ubon', on 29 September, 2020, when many pods on various stages are growing. The harvested pods were separated into immature pods (green, less than 20 mm length, but without rattle), young pods (dark green, greater than 20 mm length and a soft and elastic hull, but without rattle) and matured pods (light green, greater than 20 mm length and a hard hull). A portion of matured pods was separated into hulls and seeds after drying. The hulls sample was mixed for three replications. Dried (60°C, 72 hr) and powdered 4 g samples were extracted with 150 ml extraction solvent [acetone: water (2:1)]. The extracts were adjusted using acetone and were diluted with ethanol. One PA component present in sunn hemp seeds in large amounts is trichodesmine: its contents were quantified using LC-MS/MS (Triple Quad 6500+, AB Scie × Pte. Ltd., MA, USA).

Data were analyzed using PROC LM software (SAS Institute Inc., NC, USA) with the cultivar and maturity as fixed effects. Response variables were days from seeding to first flowering and trichodesmine content. For all statistical analyses, the SAS Add-In for Microsoft Office of AFFRIT, MAFF, Japan was used.

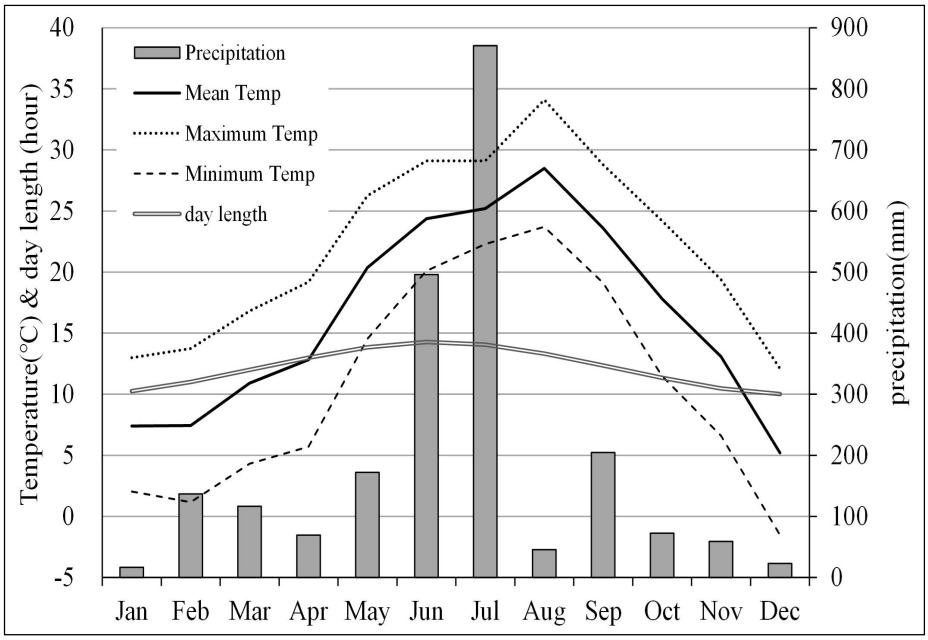

Fig 1: Temperature, precipitation and day length of the experiment year. As temperature and precipitation data during the experimental period, we used the values of weather observation equipment installed in the centre. Mean, maximum and minimum temperatures and day length were calculated as averages of the respective months. Precipitation data from 21 Jan to 4 Feb are missing.

RESULTS AND DISCUSSION

Flowering and pod mature

Although no information exists about flowering characteristics of the cultivars used in this study, flowering of the 'Kobutorisou' and 'Kurotararia' cultivars was delayed than the others. For May seeding, the first flower of these two cultivars bloomed four weeks late (Table 1). Although the remaining three cultivars bloomed at around 55 days after seeding (Table 1), none of the first flowers podded for May seeding. Flowers of sunn hemp are cross-pollinated by several bee species (Mosjidis and Wang, 2011). This is because the keel blossom structure of this flower is adaptative against bees (Westerkamp, 1997). Even in parts of India where native to sunn hemp, seeds set very little due to the lack of effective pollinated bees (Free, 1993). Cho *et al*. (2016) reported a lack of seed set because short-day sunn hemp accessions flower in fall, when these pollinators are absent. Similarly, findings from the present study suggest that pollination did not proceed well because bees were insufficient as a result of rainfall (Fig 1) and the paucity of flowers during the first flowering period (mid to late July).

For the July seeding, the difference in flowering dates between the first flowers of 'Kobutorisou' and 'Kurotararia' and the others narrowed to about one week (Table 1). These differences in flowering date among cultivars have demonstrated that the use of 'Kobutorisou' and 'Kurotararia' at July seeding or the use of another three cultivars can shorten the days to first open flower. In addition, 'Ubon' seeded in May and July flowered at 54 and 45 days from seeding (Table 1). 'Ubon' seeded in April in central Florida flowered at 92 days from seeding (Garzon *et al*., 2021). These results suggest that not only right two short-day cultivars, 'Kobutorisou' and 'Kurotararia', but also 'Nemakorori' and 'Ubon' have short-day characteristics with early maturation.

The pods grew to 20 mm length in 6-11 days from flowering. Then pods grew to maximum size (40 mm length) in only few days at the earliest (Table 1). This report is the first to describe the pod development days of sunn hemp, among with the findings that pod enlargement progressed to the maximum size within 10 days after the first flowering. Keatinge *et al*. (1998) reported that the duration from first flowering to the first mature pod of short-day sunn hemp is shorter when the temperature is higher under a long-day condition. Because the pods of July seeding matured in September, one might infer that pods grow faster in August, which was warmer than September (Fig 1).

Trichodesmine content

The content in the pods of one PA, trichodesmine, increased with pod growth and age in both cultivars (Table 2). Furthermore, no significant difference was found between the cultivars ($P<0.05$). The maximum value of trichodesmine content in immature pods (green, less than 20 mm length, but without rattle) was 13 mg kg^{-1}, but the average value was less than 10 mg kg^{-1}. The maximum value of young pods (dark green, greater than 20 mm length and a soft and

Table 1: Sunn hemp first flowring and pods growth at shortest period with six replications.

Varieties	Seed sowing in May	Seed sowing in July			
	First flowering (Days after sowing)	First flowering (Days after sowing)	Pod size†		
			10 mm length (Days after first flowering)	20 mm length (Days from 10 mm)	30 mm length (Days from 20 mm)	40 mm length (Days from 30 mm)
Nekobukira	54	51	3	3	1	1
Nemakorori	55	44	6	5	2	0
Kobutorisou	86	51	7	4	0	0
Kurotararia	85	41	4	2	0	1
Ubon	53	45	4	4	1	0

Investigate the flowers and the pods at the three to five branches which bloomed the earliest for each plot. Results shows the earliest period of six replications. † If the earliest one died on the way, then another was investigated. Therefore, the results are discontinuous.

Table 2: Trichodesmine concentration (mg kg^{-1}) in sunn hemp different maturity pods.

Varieties	Pod maturity						
	Immature pods†		Young pods‡		Matured pods§		Matured
	Mean	Maximum	Mean	Maximum	Mean	Maximum	pod hulls
Nemakorori	6.5Aa¶	8.2	25.7Aa	34.0	116.3Ba	150	2.7
Ubon	7.8Aa	13.0	22.4Aa	36.0	145.7Ba	250	11

Trichodesmine concentration were measured only the three plots (each for pods and mixed for hulls) out of six replications. † green and under 20 mm length without rattle. ‡ dark green and over 20 mm length with soft and elastic hull and without rattle. § light green and over 20mm length with hard hull. ¶ Means followed by a common uppercase letter in the row and lowercase in the column do not differ by Tukey's test (P>0.05).

elastic hull, but without rattle) was 36 mg kg^{-1}; the average value was less than 25 mg kg^{-1}. The maximum value of mature pods (light green, greater than 20 mm length and a hard hull) was 250 mg kg^{-1}; the average value was greater than 100 mg kg^{-1}. The value of matured pod hull was 2.7 and 11 mg kg^{-1}. This result well demonstrated that trichodesmine increases as the pod size grows; it subsequently increases considerably as pods mature. An earlier study demonstrated that sunn hemp seed contains over 1000 mg kg^{-1} (=2.8 µmol g^{-1}) trichodesmine (Kaneko *et al*., 2021). Ji *et al*. (2005) reported that PAs contents in sunn hemp seed were 0.8-3.8 µmol/g in accessions from several countries. In this study, the trichodesmine concentration of matured pod hull was even as high as immature pod including seed. This suggested that trichodesmine concentration increased only at the seeds in pod. Reported trichodesmine concentrations in sunn hemp stems, leaves and flowers are less than 1 mg kg^{-1} (Kaneko *et al*., 2021). The foliage and pods weights were not recorded this time, but the pods weight, especially when the first pod reaches 20 mm length, are extremely low compared to the foliage. For pods of less than 20 mm length, the maximum trichodesmine concentration was found to be 13 mg kg^{-1}. It is considered that trichodesmine is diluted in the whole plant and does not increase considerably when immature pods are included in a sample. However, if pods grow to greater than 20 mm length, not only does the trichodesmine concentration increase: the pod weight increases, which increases the whole-plant trichodesmine concentration. Therefore, for the sunn hemp harvested after 10 days from first flowering, when the pods reached 20 mm length, the trichodesmine concentration was high. The germination ability of seeds in young pods (smaller than 20 mm) has not been investigated. Seeds in immature pods might already have germination ability. One must investigate the germination ability of seeds in young pods before the incorporation of sunn hemp young pods in farmer's field.

CONCLUSION

The findings from this study well demonstrated that both day-neutral and short-day varieties with July seeding shorten the days to first flowering. And the pod enlargement progressed to the maximum size within 10 days after the first flowering. This result well demonstrated that trichodesmine increases as the pod size grows; it subsequently increases considerably as pods mature. Therefore, cutting after 10 days from flowering is effective for increasing the PA concentration in green manure.

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