Systematic Studies on *Crotalaria tetragona* Roxb. ex Andr. (Fabaceae-Crotalarieae): A Wild Relative of Sunnhemp

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Crotalaria tetragona Roxb. ex Andr. (Fabaceae) considered to be close wild relative of cultivated *C. juncea* L. (sunnhemp) occurs in the tropical Asian region. Systematic study based on floral morphology and developmental aspects of *tetragona* was attempted. The study pointed out gross structural similarity of floral parts of this species with that of *C. juncea* but differences were apparent in development of floral parts.

Key Words: Androecium, *Crotalaria tetragona*, Developmental aspects, Electron microscopy, Dimorphic stamens, Fabaceae, Keel, Pollen morphology, Standard, Stigma, Systematic study, Taxonomy, Wild relative

Introduction

Floral traits are excellent subject of study for basic and applied research on plant systematics, plant breeding and reproductive biology (Hoc *et al.*, 2003; Ford and Johnson, 2008; Koul and Bhatnagar, 2007; Parsons and Luise, 2006). Data from floral morphological studies have provided baseline information on phenology, breeding, pollination biology, phylogeny, crop genepool study and plant systematics, patterns of evolution within species and rates of divergence between species (Etcheverry *et al.*, 2008; Polhill, 1982; Striton, 1981; Uga et al., 2003).

Flowering plants display extraordinary diversity in morphology of floral traits, yet the functional significance of these variations is not well understood in many important taxa. Studies on variation in floral morphology have thrown light in understanding the pattern of adaptability in flowering and fruiting (Etcheverry et al., 2008; Jacobi et al., 2005; Patterson, 1982). The study of morphological structure of complex flower has helped in understanding the mating system, compatibility and seed set in members of Fabaceae including *Crotalaria* (Free, 1970; Kalisz et al., 1999; Etcheverry, 2001b; Etcheverry and Aleman, 2005; Jacobi *et al.,* 2005).

Despite wide knowledge available on various aspects of floral biology of Fabaceae, the genus *Crotalaria* is least worked taxa for systematic potential especially for many indigenous taxa (Pandey and Nayar, 1994; Ansari, 2008). Morphology of floral parts has provided data of relevance to taxonomy in different species of *Crotalaria* (Pandey and Nayar, 1994; Polhill, 1982). *Crotalaria juncea* L. known as sunnhemp, a source of the oldest cultivated fibre from Indo-Pakistan region (Watt, 1989) does not occur wild. *Crotalaria tetragona* Roxb. ex Andr., presumably a wild relative of *C. juncea* is a tall shrub distributed in tropical Asia. This species occurs as wild and semi-domesticated population and reported for ethnobotanical use (Bhatt et al., 2009). It has gross morphological resemblance to *C. juncea* (Babu, 1977; Pandey and Nayar, 1994) but can be distinguished from the latter in general habit, tetragonous stem, linear-lanceolate leaves, longer pods (3.5x1.4 cm as compared to *C. juncea* with 2.1x1.2 cm) and larger seeds (0.51x0.43 cm as compared to *C. juncea* with 0.4x3.8 cm). Overlapping of many of the floral traits with *C. juncea* was suggestive of a close relationship between the two taxa and, thus, needs investigation on systematics (Babu, 1977; Baker, 1876). Earlier studies on *C. tetragona* have been carried out with respect to floristics (Baker, 1876; Babu, 1977; Ansari, 2008), anatomy (Mangotra, 1991), chemotaxonomy, karyobiology (Mangotra and Bhargava, 1989) and phenetics (Raj *et al.,* 2011). The present investigation was aimed at study of *C. tetragona* with respect to morphology and development of the floral parts more prominent in corolla and androecium characters to support plant taxonomy and systematic of the taxa.

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Materials and Methods

Seeds of *C. tetragona* collected from disturbed habitat of Kolasib district in Mizoram, India were grown in the experimental pots in the net-house at National Bureau of Plant Genetic Resources (NBPGR), New Delhi. The studies were undertaken for three consecutive years (2009, 2010 and 2011). The seeds were sown in earthen pots of 40 x 35cm size filled with standard potting mixture (containing sand, soil and farmyard manure in equal proportion). Watering was done every day once during July-October and at weekly interval during October-November. Fertilizers were applied with different doses of NPK (30:10:30) during vegetative phase (August-October) and NPK (10:30:10) during flowering season (October-December). Plants were continued to grow in the net-house until they started flowering after 3 months of germination. For floral observations inflorescence from middle internodes and flowers in the mid season were used. The data on floral morphology and structural developments were recorded from 9.0 am to 5.30 pm from mid October to mid December months continuously. Inflorescence length and number of flowers were recorded on primary and secondary branches during the entire experimental period. A total of 50 plants were selected for recording the observations on selected parameters from stages of bud initiation to flower senescence during peak flowering period (July-November). Estimates of number of flowers per plant were made by counts of total number of inflorescence per plant (using 50 plants) and flowers per inflorescence (50 inflorescences). To relate floral morphology and structural development within the inflorescence 50 flower buds of same size were marked (tagged) and observed thrice a day (9, 13 and 17 hours). Developmental stages of the flower were marked arbitrarily as six stages (day 0 to day 5) to record, correlate and interpret results (Table 1). Voucher specimens were dried according to standard herbarium techniques and deposited in the National Herbarium of Cultivated Plants (NHCP), NBPGR, New Delhi and herbarium of the Botanical Survey of India, Sikkim Himalayan Circle, Gangtok.

Data on inflorescence length, number of inflorescence, inter-floral distance, number of flowers per inflorescence and mean numbers of flower opened per day, flower size, standard size, wing size, keel size and ovary size were recorded and mean values calculated ± standard error (S.E.). Observations on weather data were recorded periodically for temperature (minimum and maximum average value) and rainfall pattern during course of study (meteorological data for 2009-11) and data presented as mean value. Pollen of the two species were collected from ten selected flowers from dehiscing anthers of two types of stamens i.e. short (SS) and long stamens (LS) separately at different growth stages (freshly opened to fully matured flowers) (day 2 onwards) and studied for micro-morphological characters. Floral morphology of the taxa was studied with freshly harvested selected buds/flowers and data recorded for morphology of separated floral parts in the inflorescence for characters. Measurement of floral parts was done to the nearest 0.5 mm. Androecium size was measured at different growth stages and calibrated image captured using Stereoscopic Zoom Microscope (SMZ1000/800; 10X-1000X magnification). Pollen and stigma structures were fixed in 2.5 per cent glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) followed by ascending series of alcohol and allowed to air dry (Dykstra, 2003). Air dried samples were fixed on the SEM specimen stubs with a double-sided adhesive carbon tape and placed on the revolving discs of JEOL fine coat ion sputter (JEOL JFC 1100) for coating with gold. Each sample was uniformly coated with 20-30 nm thick gold. Images were taken under Scanning Electron Microscope (JEOL-JSM 840A) operated at accelerating voltage of 5-10 Kv. Structural details of micromorphology and dimension of pollen of two types of stamens were recorded. Observations on different growth stages of stigma and pollen were examined using compound microscope (Olympus CH40; 100-1000x magnification).

Table 1. Stages (days to open) of flower development recorded in *C. tetragona*

Stage (days to open)	Relative time to flower opening (hours)	Development recorded
0	-48	Bud primordial development
1	-24	Bud elongation with wide splitting of calyx lobes
2	0	Initiation of flower anthesis
3	12	Corolla half opened
4	24	Corolla fully opened with standard erected at right angle (flower fully developed); pollen burst from two types of anthers
5	48	Flower maturity with maroonish yellow corolla (initiation of flower senescence and pod primordia development)

Results

Selected seeds were grown for three consecutive years in the months of July-August (2009-2011) (average maximum and minimum temperature 30.67 and 20.83° C; rainfall 3.87 mm). The first blossom appeared in end of September and continued till December. However, peak flowering was recorded during October-November. Floral characters recorded for six arbitrary stages from initiation of bud to pod initiation are given in Table 1. The observations showed that: a) Bud to flower senescence took 72-90 hours (over five days); b) Anthesis started in the morning at 9 hours (second day onwards) and completed by 24 hours (on fourth day); c) Flowers showed a distinct characteristic corolla (standard and keel); and d) Stamens had dimorphic anthers and showed distinct morphology and developmental aspects.

Structures and developmental aspects

The total number of flowers produced per plant were 95.23±0.257 (N=50). The inflorescence was laxed with terminal and axillary racemes, flowers arranged in acropetal maturation (base to apex). Terminal inflorescence was longer (260.3±1.269mm; N=50) than the axillary inflorescence (122.45±0.61mm; N=50). The terminal ones appeared during the first month of flowering followed by axillary inflorescence that were more in number more towards the second half of the December in the experimental period. The flowers were distantly arranged with inter-floral distance of 25.66±0.096 mm (N=50) in terminal and lateral inflorescences. Single plant produced 7.16±0.165 (N=50) inflorescences and total number of flowers per inflorescence was 13.30±0.35 (N=50). Mean number of flowers opened at a time was 2.8±0.26 (N=20) per inflorescence.

The bud primordia of *C. tetragona* increased from 3-9 mm to 20-25 mm within 24 hours before flower opening (stage 0; Fig.1a). The mature flower buds are beaked, lemonish-green, linearly elongated during one day before flower opening (stage 2; Fig.1b). The flower is beautiful, attractive lemon yellow colour, zygomorphic, protandrous and 39.60±0.06 mm (N=20) long on drooping peduncle (clavate shaped, 1.4 cm; 1x3-1x4 mm). It was narrow (1mm) at base broadening (4mm) towards top and beared three markings, two on each side and one on the top. The calyx is bilipped and corolla has wing-keel complex with erect standard (vexillum) on the upper side (stage3-4; Fig.1c-d).

The bracts were setaceous (two, each 3 mm long), minute, located at base of peduncle; bracteolate (two; each 2.109 mm long). Calyx is gamosepalous, five sepals, lemonish green, soft brown, tomentose, with five teeth of variable length; two upper laterals are connate at base (20x3 mm) resting on the standard, two lower laterals and a lower median united laterally from base to half of the length (21x4 mm). Corolla is slightly exserted, size of standard 27.10±0.1 mm x 25.45±0.069 mm (N=20), suborbicular, apex mucronate, turned backwards in completely open flower (stage 4 onwards), spotted or streaked, a little wider than length, maroonish, ribbed on maturity; wings are ovate-oblong (20.1±0.1x10.20±0.06 mm, N=20), spatulate, claw thick (ca 3 mm), dull lemon yellow with unique curvature; keel is lemon (20.50±0.1 mm x15.05±0.1 mm; N=20), outcurved, conical on lower half, beaked with twisted tip; ovary subsessile (7.50±0.02 x 2.6±0.01 mm; N=20), tomentose, 10-18 ovules, stigma hairy; gynophore (a short stalk holding ovary; 4mm) and the furrow forming a chamber for nectar accumulation. The wing and the keel on the dorsal mid rib had thicker dark maroon coloured line and other radiating lines and folds on the lower half between veins (Fig. 3 d, e, f; see arrow). The basal part and blades of keel are fused on the lower margin upto tip by a genuine fusion. Terminal end of the keel is straight in bud but gradually twists leaving a small pore (*ca* 1 mm aperture) opening at the tip in mature flower (Fig. 2e,f). The bilipped corolla at base of gynophores, keel, ovary and staminal furrow formed a cavity to facilitate access by insect pollinators.

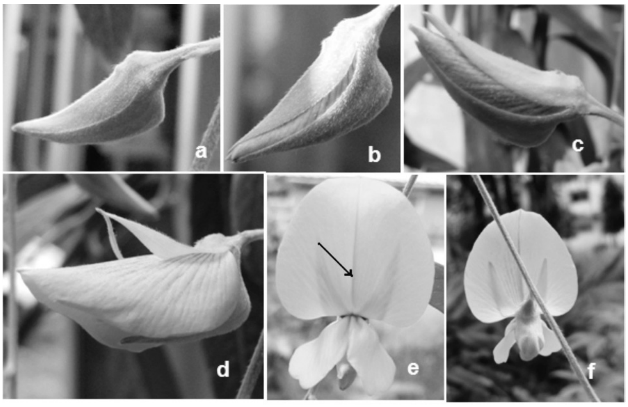

Fig. 1. (a-f) Stages of flower in *C. tetragona* a. One day old flower bud. b. Initiation of splitting of the calyx. c. Calyx splitting upto peduncle point (bilipped), lower half with three lobes and upper with two lobes. d. Corolla half open with calyx connate only at tip. e. Standard erect in fully opened flower with prominent wings, drooping on lower side and keel in the middle. f. Back side view of fully opened flower showing upper two lobes free and three lower lobes of calyx fused (for stages 0-5 refer to Table 1).

Androecium

Androecium is monoadelphous, composed of ten dimorphic, basifixed anthers with short stamens (SS) alternating with long stamens (LS). Short stamens (SS) have shorter filaments but longer anther lobes and dehisce during the 12 hours (initiation of anthesis; stage 3). They alternate with five long stamens (LS), having longer filaments but round anther lobes and dehisce 24 hours after anthesis (stage 4 onwards). The two types of anthers dehisce longitudinally and showed differential growth. However, the dimensions of the pollen grain from two types of stamens did not show any significant difference.

The filament of both types of stamens remained turgid up to third day (stage 4) but afterwards floral senescence started with initiation and twisting of filament of SS (Fig 3d). Pollen was tri-colpate, 3-zonocolporate with microperforate to faintly reticulate exine (Fig. 2). The shape of pollen grain was prolate and size 34.3 x 14.3 µm (Fig. 2).

Fig. 2. a. Pollen grains of *C. tetragona.* b. Enlarged pollen grains with markings on exine surface. c. Microperforate ornamentation on exine wall

In young buds, SS have larger primordia than the LS (Fig. 3a). At the time of flower anthesis (stage 2), filaments of the SS are slightly longer than the entire LS (Fig. 3b). Filaments of all the stamens join into a furrow surrounding the ovary. From second day onwards there is significant increase in filament length of both types of stamens; the filament of the LS supersedes the SS (stage 3). By third day, both the filaments and anthers of the two types showed further development: the filament of SS grows from 1.85 mm in the bud to *ca* 9.55 mm in 24 hours after anthesis whereas the LS grew from 2.0 mm to 12.8 mm in the same period and further more up to 14.15 mm (Fig. 4). Dehiscence of anthers took place on the day 2 in SS and on day 3 onwards in LS, forcing the mass of pollen progressively into the conical and twisted tip of the keel around style. The size of anther lobes of SS was five time bigger than that of the SS and produced more quantity of pollen grains that remained viable for longer floral cycle as compared to that of the LS. The filament of LS grew around the stigma and shed all pollen leaving shrivelled anthers.

In *C. tetragona* at stage 1 filament of the two types started elongating, but anthers were not above carpels. At the stage 2 filaments partially elongated, anther beginning to emerge body, but not above. When after 12-16 hours at stage 3 filaments were elongated, anther above carpels tops but anthers did not dehisce. Lastly at stage 4 onwards, filaments were fully elongated, occur parallel to the body of style, and both anthers showed anther dehiscence (SS bursts first followed by LS). At stage 5, the anthers were abscised with senescence of other flower parts such as sepals, petals, etc.

Gynoecium

The gynoecium of *C. tetragona* had short gynophores at bud stage. The ovary is fully covered with long, pubescent and trichomatous golden hairs. During the floral cycle, style at the base formed a notch (more notched when senescence followed) from straight (180° at 0 stage) to an acute/ right angle (80-90°) at stage 4 (Fig. 3a-c). Along the inner side of style long golden hairs occurred uniformly. A tuft of peristigmatic whorl of hairs surrounded the stigma (Fig. 4a-b). The stigma

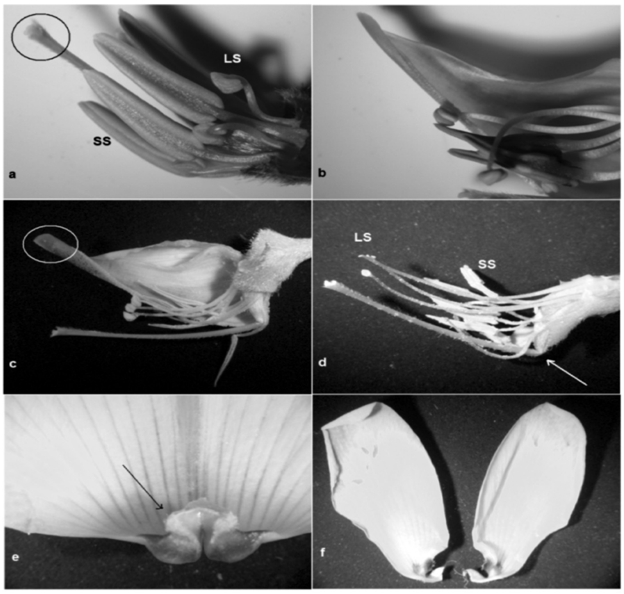

Fig. 3 (a-f) Floral parts of *C. tetragona* : a. Bud showing SS longer than the LS surrounding the style at straight angle, stigma (encircled). b-d Elongation of LS, twisted keel in flowers at stage 3, 4 (encircled), notched style (arrow); LS reaching near the stylar end, see hairy stigma and style covered with pollen. e Standard in the close up; see small claws (arrow), mid rib and radiating veins. Wings with coloured markings and deep curvature; periodic change in angle of style (from straight to 90 degrees) and hairy stigma (see in stages a-d)

was obliquely terminal and relatively small (386 µm ± 5.64, N=10) (Fig. 4a). Stigmatic surface was composed of two types of epidermal papillae, the longer ones in centre showed increase in length at different stage of gynoecium development (Fig. 4 c). Gynoecium showed a growth from 12 mm at bud stage to 24 mm full maturity (Table 2).

Discussion

In the present study, we investigated the floral morphology with special emphasis to the structure of androecium and gynoecium development in the entire cycle (bud-flower senescence) of *C. tetragona*. These floral structures and developmental stages were distinct when compared with related *C. juncea* (Pandey and Nayar, 1994; Pandey *et al.,* data unpublished). Despite of much resemblance in flower morphology of the two taxa, structure and development of anther and stigma were very distinct. The key observations on the investigation carried out are discussed below in terms of application in taxonomic and systematic study.

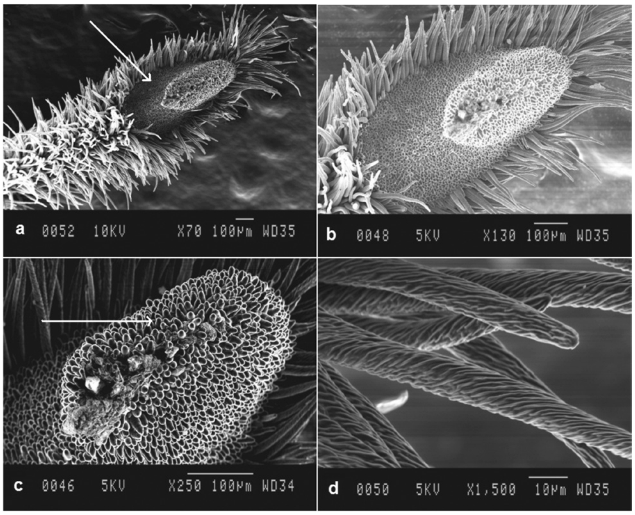

Fig. 4. SEM photomicrographs of the stigma of *C. tetragona* (a-d). a Stigma at stage 2 showing peristigmatic hairs (arrow). b Closer view of stigma showing distinct long and short epidermal papillae on the surface. c Stigmatic surface with epidermal papillae in enlarged view (arrow). d Peristigmatic hairs in closer view

Table 2. Average growth and development of androecium and gynoecium of *C. tetragona* at different stages

Stage (days to open)	Relative time to flower opening (hours)	Filament of short stamen (SS) mm	Filament of long stamen (LS) mm	Length of gynoecium (mm)
0	-48	1.85	2.0	12.40
1	-24	3.7	2.3	13.05
2	0	5.5	10.1	21.85
3	12	7.0	11.1	22.90
4	24	9.55	12.8	23.65
5	48	9.55	14.15	24.70

General Floral Morphology

Floral morphology and study of development of parts have provided a better understanding of relationship and evolutionary pattern in different taxa under order Fabales (Bellow *et al.,* 2007; Etcheverry *et al.,* 2008). The investigation made on morphology and floral development revealed the complex structure of *C. tetragona* flower exhibiting sequence of changes in floral parts *viz.* petals, androecium and gynoecium (Pandey and Nayar, 1994; Ansari, 2008).

Characteristic floral morphology with deeply cleft two lipped (bilipped) calyx, as long as corolla, protracted downwards, keel tip spirally twisted from inception, corolla appendages restricted to the blade, standard marked with reddish-brown lines, and style curved (notched) with conical lower half have shown the similarity with *C. juncea.* Similar observations were reported by other workers (Ansari, 2008; Pandey and Nayar, 1994). These observations supported taxonomic placement of *C. tetragona* under section Calycinae. Data on floristic studies and presence of flavonoids have also supported placement under section Calycinae (Polhill, 1982; Mangotra and Bhargava, 1989; Ansari, 2008).

Inflorescence, Calyx and Corolla Structure

Inflorescence of *C. tetragona* was distinctly laxed with more number of flowers. Inflorescence were observed to be terminal or lateral, lax with long peduncle and 120-360mm long as recorded by other workers (Singh *et al.,* 2000, 2002; Ansari, 2008). In general in *Crotalaria* species the inflorescence are terminal, lateral or both, rarely capitate (Ansari, 2008). The flowers were arranged in acropetal succession of opening that increased chances of pollination under unsuitable environment. This observation was also recorded in many Papilionaceous flowers (Suzanne Koptur, 1983; Etcheverry, 2001a; Caruso, 2006). Slow pace between the opening of consecutive flowers (2-3 flowers opened at one point of time) in contrast to related species *C. juncea* where the time gap between the consecutive flower was lesser (at one time almost half the flowers of a mature inflorescence were opened) were distinguishable feature (Pandey *et al.*, data unpublished). These differences could be related to distribution of two species.

Development of Floral Parts

The flower of *C. tetragona* showed a typical keel blossom type of corolla with floral morphological adaptations such as bright colour of petal and wings structure, presence of nectar, and odoriferous flowers. Similar observations pointed out in other taxa by Westerkamp (1997) were indicative of adaption for pollinators. In many Papilionaceae including *Crotalaria micans, C. incana, C. pumila* and *C. stipularia* micro-morphology of wings exhibited modification into a pocket-like sculpturing as footholds during the pollinators foraging activities (Etcheverry, 2001c; Solomon and Purnachandra, 2006). In this species standard erect backwards in fully opened flower (stage 4) with prominent markings on the wing, drooping on lower side support the sitting of pollinator and keel in the middle which coincides with its body (Fig. 1e). In *C. juncea* the flowers were not drooping, and in fully opened flower the standard turned at right angle position. In various members of tribe Vicieae the flowers showed distinct structural morphology of androecium and pistil (Gunn and Kluve, 1976).

Androecium

In Fabaceae development of keel (gradual twisting) and androecium were suggestive of economic utilization of pollen that co-evolved with the pollinators (Hoc et al., 2003; Prenner, 2004; Etcheverry *et al.*, 2003; 2008). In *C. tetragona* structural changes linked to differential maturity of androecium and pollen grains in two types of stamens were also observed by Etcheverry *et al.,* (2003). These changes coincided with corolla growth (stage 3) may be adaptive features that suggested of pollinator-mediated efficient mechanism, especially under unsuitable environmental conditions. Despite of morphological similarity of anthers of *C. tetragona* with that of *C. juncea*, in the latter species long anthers burst much before the opening of flower at 2 stages (Pandey et al.; data unpublished).

The pollen grains from both types of anthers were structurally similar as observed by many authors (Etcheverry *et al.,* 2003; Ansari, 2008). Pollen grains were prolate in shape, tri-colpate and 3-zonocolporate with faintly reticulate exine. Nair (1965) has reported 3-zonocolporate type of pollen grains being most common among family Fabaceae.

Gynoecium

Stigma architecture provides data for taxonomic delimitation and classification at infra-family levels in various taxa under Fabaceae (Ghosh and Shivanna, 1982; Venkata Lakshmi and Shivanna, 1985; Shivanna and Owens, 1989; Owens and Lewis, 1989, 1996; Etcheverry et al**.**, 2003). Some closely related species are distinguishable on the basis of pollen brush type of style and thus have proven taxonomic utility (Lavin and Delgado, 1990; Kang and Zhang, 2009).

In *C. tetragona* the stigmatic surface showed epidermal papillae in the centre forming highly receptive area (Fig. 4). Similar structure was described in *Crotalaria micans* by Etcheverry et al. (2003). A tuft of peristigmatic whorl of hairs surrounded the relatively small stigma, gynoecium forming a typical notch at the place of ovary and style junction and trend of hairiness along the inner side of style were characteristic features of this species. *C. juncea* could be distinguished by shorter peristigmatic hairs surrounding small stigma with protruding tip and thin hairs present only on the inner side of style. Similar observations were helpful in distinguishing different taxa under *Crotalaria* (Pandey and Nayar 1994; Ansari 2008).

Conclusion

On the basis of above observations we might interpret the two taxa, *C. tetragona* and *C. juncea* closer as pointed out by several workers (Babu, 1977; Ansari, 2008; Pandey and Nayar, 1994). In various legume taxa similar observations have been reported by Tucker (2003). Currently there is insufficient information on floral aspects of related taxa especially in genus *Crotalaria* to evaluate them for taxonomic interpretation. The study could be further extended for larger crop genepool of *Crotalaria* from the Indian subcontinent showing tremendous diversity and endemism.

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