

Is stem twining form of *Basella alba* L. a naturally occurring variant?

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A naturally occurring circumnutating stem form of *Basella alba* L. has been characterized in the background of two procumbent stem forms of *B. alba* L., cultivated commonly as leaf vegetable. The difference between the procumbent and circumnutating stem forms was quite distinct particularly in terms of biomass since the circumnutating form showed reduced laminar area and weight but with long internodes and minor stem circumference, which is otherwise perfectly suited for circumnutation. Though there were no cytological differences between the three materials under study, the loss of certain unique DNA fragments in the genome as revealed by amplified fragment length polymorphism (AFLP) and proteins in the proteome as revealed by two dimensional gel electrophoresis were obvious in the circumnutating stem form. This loss probably contributed to change in function of twining in the circumnutating form; and this phenotype plausibly is a stable variant of the more common procumbent form of *Basella*.

Keywords: AFLP, *Basella*, procumbent and twining stem forms, two dimensional gel electrophoresis, variant.

BASELLA (*B. alba* L. and *B. rubra* L.), also known as Malabar spinach, Indian spinach, Ceylon spinach and vine spinach is cultivated as an annual leaf vegetable, which is grown in almost all parts of India, tropical Asia and Africa. Young shoots and leaves of *Basella* are a rich source of calcium, iron, and vitamins A and C. Red cultivars contain a colouring matter in leaves, stems and ripe fruits which can be used for colouring food. The purple dye of *B. alba* has also proved to be fairly suitable as an indicator in weak acid/strong base titrimetry¹. Because of its mucilaginous nature, *Basella* leaves are used as poultice. Juice of leaves is prescribed for treating constipation, especially in children and pregnant women².

Basella (*B. alba* L. syn. *B. rubra* L.) originated in Asia and more particularly in India. It is a monotypic genus of the family Basellaceae. There is only one species. There are three main types under cultivation. The most common type has dark green, oval or nearly round leaves, which is *B. alba*. The other less popular type, often referred to as *B. rubra*, has round oval leaves and red stem. The third type less commonly grown, has cordate shaped dark green leaves and is referred as *B. cordifolia*. Chromo-

some number of *B. alba* is $2n = 48$ and of *B. rubra* is $2n = 44$ (ref. 3).

Bailey described *Basella* as a genus with narrow leaves, whitish flowers on long peduncle; spikes arranged in very close clusters⁴. Haines described *B. rubra* as a fleshy twining, much branched herb with ovate shiny and rather fleshy leaves and small sessile pinkish flowers on distant spikes⁵. The initial study towards characterization of two stem forms of *Basella* revealed that the procumbent stem forms have distinct colouration, viz. green and red; and the other stem form has the first priority – circumnutation⁶. It involves the circling of the shoot tip as it attempts to find a support. The twining stem form, which has sometimes been referred to as another species, *B. cordifolia*, appears to be a distinct variant of the more common and often cultivated procumbent stem forms. The present paper attempts to substantiate the aforesaid assumption by genomic and proteomic approaches.

Two stem forms of *Basella* (*B. alba* L. syn. *B. rubra* L.) viz. twining (Figure 1a) and procumbent (both green and red) (Figure 1b) were used as materials for the present study. Standard leaf (considering basal, middle and apical portions of individual leaf of three developmental stages) and stem parameters were studied for morphological characterization of three plant materials.

Fresh stem (procumbent/twining) portions of matured plants were subjected to serial hand-sectioning for obtaining transverse sections followed by conventional double staining method for vascular plants. Meiosis was studied from temporary smear preparation of pollen mother cells after fixation of flower buds in propionic acid and absolute alcohol (1:3) and stained in 1% propiono-carmin. For scanning electron microscopy, flower buds of appropriate stage were collected and isolated pollen grains were directly subjected to sputter coating with gold (7 mA). Photographs were taken under FEI Quanta 200 MK2 Scanning Electron Microscope.

Genomic DNA was extracted from 10 days old germinated seedlings using CTAB method followed by a step of purification with Qiaquick DNeasy plant minikit (Qiagen). Restriction digestion of genomic DNA (300 ng) was carried out using *EcoRI* and *MseI* followed by ligation of oligonucleotide adapters compatible with these endonucleases following the protocol of Invitrogen. Pre-selective polymerase chain reaction (PCR) amplification was performed using the amplified fragment length polymorphism (AFLP) kit of Invitrogen. The PCR profile for the pre-selective amplification was: 94°C for 2 min, followed by 20 repetitive cycles of denaturation at 94°C for 30 s, annealing at 56°C for 1 min, and extension at 72°C for 1 min followed by a final extension at 72°C for 7 min. All samples were stored at 4°C following amplification on a Thermal Cycler (MJ Research). The amplified products were then considered for selective amplification with γ -³²P ATP-labelled and unlabelled *MseI* primers having three pre-selected nucleotides at the 3'-end (first

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31 of total 64 possible primer combinations of Invitrogen AFLP kit). Selective amplification was performed following the protocol of Invitrogen. The PCR cycle of initial 11 cycles was: denaturation at 94°C for 30 s, annealing at 65°C for 35 s, with touch down of 0.7°C per cycle, extension at 72°C for 1 min followed by next 25 cycles: denaturation at 94°C for 1 min, annealing at 58°C for 35 s and extension at 72°C for 1 min⁷. After selective amplification, 20 µl of the PCR products was subsequently diluted with equal amount of formamide dye (98% v/v formamide, 10 mM EDTA, 0.025% w/v bromophenol blue, 0.025% w/v xylene cyanol) and the products were heated for 3 min at 90°C and placed immediately on ice. Products (1.5–2.0 µl) were resolved in (6% polyacrylamide) sequencing gel at 1600 V for 2½ h. The gel was dried in a vacuum drier at 80°C for 1 h and 15 min and exposed to X-ray film (Kodak). Photographs of AFLP profiles were digitized and analysed with Bio-Rad Quantity One, 1-D Analysis Software, Version-4.6.2.

Total protein was extracted from appropriate stem portions (procumbent/twining) of matured plants using ReadyPrep™ Protein Extraction Kit, quantified by RC DC Protein Assay kit and was subsequently purified by ReadyPrep™ 2-D Cleanup kit of Bio-Rad. The protein solutions (containing 70 µg protein) were loaded on dry IPG strips with reswelling buffer of pH gradient 3–10 NL (Immobiline dry strip, 13 cm, GE Healthcare). The strips were then re-hydrated overnight, and isoelectric focusing was performed according to the following voltage gradient: 50 V for 4 h, 50–500 V for 30 min, 500 V for 2 h, 500–2000 V for 1 h 30 min, 2000 V for 2 h, 2000–3500 V for 1 h and finally 3500 V for 11 h for a total of 38,000 volt hours. Equilibration of gel strips was performed in an sodium dodecyl sulphate (SDS) equilibration buffer with dithiothreitol (DTT) for 15 min and then with iodo-

acetamide for 15 min. Second dimension gel electrophoresis was performed on 10% polyacrylamide gel (PAGE) followed by standard protocol of silver staining method for detection of protein spots. Gel images were digitized with a Bio-Rad Versa Doc.

Statistical analysis of the data was made with MINITAB Data Analysis Software; Release 6.1.1 – standard version. Dendrogram on the basis of results from AFLP analysis was computed from pairing affinity (percent similarity) values (calculated in Bio-Rad Quantity One, 1-D Analysis Software, Version-4.6.2) by UPGMA method (Unweighted Pair Group Method with Average)⁸ using the statistical package EASE⁹. The distances presented here are euclidean.

Results of morphological characters of leaf and stem of the two procumbent stem forms revealed general similarity, the only difference being their colouration, either green or red. Lamina shape and quantitative characters were identical as reflected from the critical difference values between them after performing one way Analysis of Variance (ANOVA; Table 1). These two procumbent stem forms though near-identical in terms of quantitative characters but both showed significant differences ($P < 0.05$) with the twining stem form, which was characterized by leaves of much reduced dimensions and weight (Table 1). Apart from the leaf parameters, the stem characteristic of the twining form with significantly long internode and very minor stem circumference in comparison to both the procumbent forms with higher biomass were significant observations (Table 1).

Histology of the two stem forms was conspicuously different in their outline and vasculature (Figure 1 c and d). Numerous vascular bundles were localized in a peripheral ring in case of the twining form (Figure 1 c). No essential difference was observed between the two

Table 1. Comparison of morphological characters (mean \pm SE in case of quantitative character, $n = 9$) of two stem forms of *Basella* (*B. alba* L. syn. *B. rubra* L.) viz. procumbent (both green (BG) and red (BR)) and twining (BT). Standard errors (SE) of means and critical differences (CD, at 5% level) have been provided at the bottom of the table after performing one way Analysis of Variance (ANOVA)

		Characters										
		Leaf						Stem				
		Lamina					Petiole length (cm)	Stomata			Internode	
Materials	Shape	Length (cm)	Breadth (cm)	Area (cm ²)	Fr. wt/ leaf (g)	Dry wt/ leaf (g)		Length (µm)	Breadth (µm)	Frequency	Length (cm)	Circumference (cm)
BG	Ovate	14.7 \pm 0.36	9.45 \pm 0.21	139.2 \pm 6.14	16.26 \pm 1.48	1.05 \pm 0.11	3.64 \pm 0.15	37.63 \pm 0.75	22.96 \pm 0.63	7.06 \pm 0.14	4.69 \pm 0.23	6.30 \pm 0.04
BR	Ovate	14.4 \pm 0.37	9.57 \pm 0.24	137.8 \pm 6.26	17.25 \pm 1.16	0.89 \pm 0.08	2.78 \pm 0.19	39.76 \pm 0.58	21.15 \pm 0.96	5.83 \pm 0.16	4.84 \pm 0.24	6.47 \pm 0.29
BT	Cordate	7.67 \pm 0.18	6.27 \pm 0.13	31.99 \pm 1.11	2.23 \pm 0.12	0.15 \pm 0.01	1.97 \pm 0.41	40.30 \pm 0.58	29.30 \pm 0.59	7.00 \pm 0.16	7.91 \pm 0.39	0.76 \pm 0.01
SE (mean)		0.365	0.182	0.952	0.242	0.046	0.07	0.221	0.064	0.093	0.122	0.067
CD (5%)		1.26	0.63	3.29	0.84	0.16	0.24	0.76	0.22	0.32	0.42	0.23

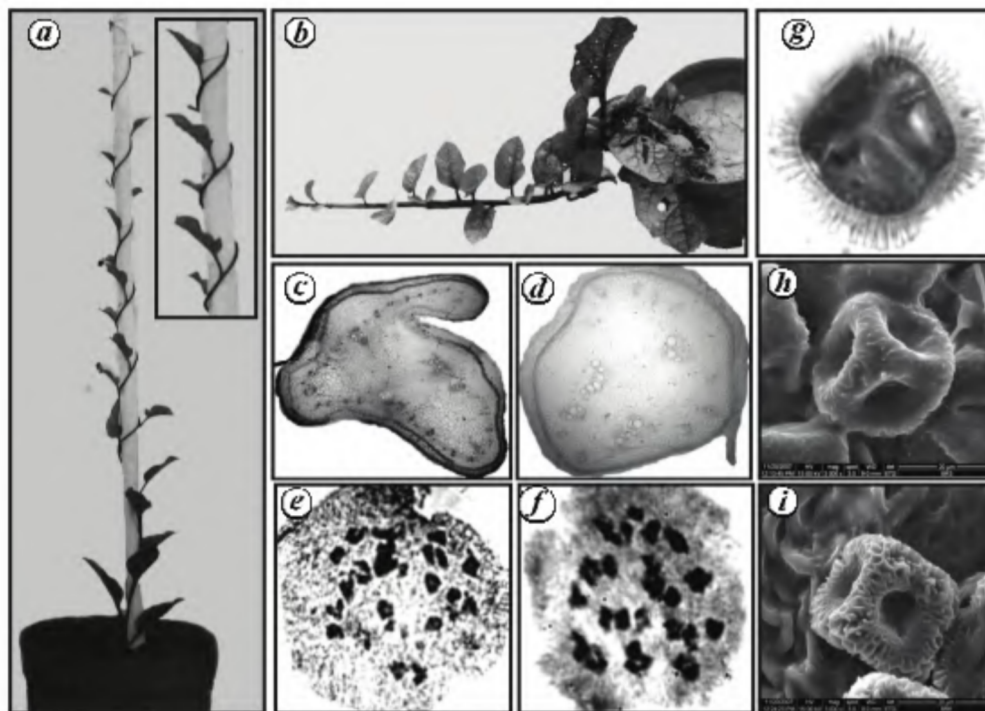


Figure 1. Twining (a) and procumbent (b) stem forms of *Basella*. The diameter of the pot is 25 cm. Cross-sections of stem of *Basella*, twining (c) and procumbent (d) ($\times 100$). Meiotic metaphase of twining (e) and procumbent (f) stem forms of *Basella* ($\times 1000$). Pollen grain of *Basella* under light ($\times 1000$) (g) and scanning electron microscopy ($\times 3000$) (h, i).

procumbent forms, i.e. green and red, hence a section of only the red form was provided (Figure 1 d). The haploid chromosome number of both the stem forms was found to be 24 (Figure 1 e and f). No multivalent formation was recorded in any form of *Basella*. Non-disjunction was found to occur in a few pollen mother cells. Pollen grains of three plant materials studied both with light (Figure 1 g) and electron microscopy (Figure 1 h and i) failed to demarcate any unique features. Pollen grains of all the materials possessed the following characteristics: 6-rugate, dice shaped grain, length of sides $\pm 32.5 \mu\text{m}$, L/B of rugae $\pm 14.0 \times 4.0 \mu\text{m}$; exine $\pm 7.0 \mu\text{m}$, sexine $\pm 6.0 \mu\text{m}$ thick, sexine more thick in corner. Exine was considerably reticulate, reticulation being finer and dense surrounding the aperture and coarser in the corners.

AFLP fingerprints of genomic DNA of two stem forms of *Basella*, viz. procumbent (both green (BG) and red (BR)) and twining (BT) (Figure 2) derived from the first 31 of total 64 possible primer combinations were analysed with suitable software. Of the 31 primer combinations, only 11 generated significant polymorphic differences among the plant materials under the study, though overall percentage similarity either between BG/BR, BG/BT and BR/BT was considerably high in most of the primer combinations (Table 2). Identical AFLP profile was obtained in the rest of the 20 primer combinations, hence, not con-

sidered for further statistical analysis leading to computation of hierarchical dendrogram. Unique AFLP derived DNA fragments, which either appeared or disappeared in BT with respect to BG and BR, were noted in the 11 primer combinations and the size of those fragments was calculated. Of the total 29 such fragments of interest (Figure 2 b–s), 18 were absent in the profiles of BT with respect to BG and BR (size range between 73 and 281 bp), whereas 11 new fragments were noticed in BT with respect to BG and BR (size range between 159 and 372 bp) (Table 2).

Dendrogram was computed on the basis of pairing affinity values derived from AFLP analysis. Cluster analysis revealed splitting of three plant materials under study into two, BG and BR, two procumbent forms forming one cluster, being similar at a distance of 2.28; whereas BT, the twining stem form was distinctly distant from the procumbent stem forms, three being similar at a distance of 2.64 (Figure 3). No essential difference was observed between the protein profiling of two procumbent forms, i.e. green and red, hence the result of the red form was only provided (Figure 4 a) with respect to the profile of twining stem form (Figure 4 b). Of the large number of protein spots, maximum were identical in both BT and BR, which can conveniently be designated as the common protein spots (marked in Figure 4 a and b);

Table 2. Results of amplified fragment length polymorphism (AFLP) analysis of two stem forms of *Basella* (*B. alba* L. syn. *B. rubra* L.) viz. procumbent (both green (BG) and red (BR)) and twining (BT)

Primer combinations	Percentage of similarity between			Size of unique DNA fragments (bp) in BT with respect to BG and BR	
	BG × BR	BG × BT	BR × BT	Appeared	Disappeared
E-AAC/M-CAG	88.2	77.4	78.4	250	281
E-AAC/M-CAT	93.7	85.7	85.8		276, 262, 194
E-ACA/M-CAA	65.2	85.9	59.5		92, 74
E-ACA/M-CAC	65.5	74.4	58.9	201, 196	
E-ACT/M-CAA	45.8	47.1	59.6		158, 156, 154
E-ACT/M-CAC	77.9	69.4	70.7	171	166
E-ACT/M-CAG	51.7	37.6	39.4	183, 321	164, 236
E-ACT/M-CAT	80.2	77.2	69.8		278
E-ACT/M-CTA	82.2	69.2	65.0	246, 192, 159	281, 194, 161
E-ACT/M-CTC	65.9	74.6	56.4	372, 325	73
E-ACT/M-CTG	80.7	80.3	81.4		130

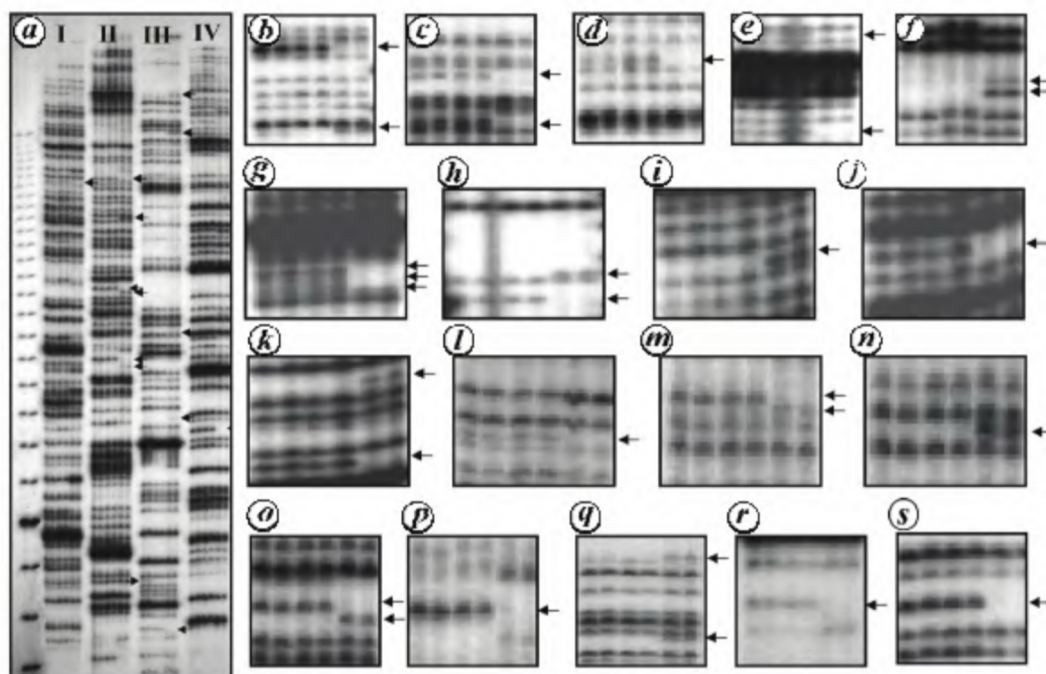


Figure 2. Amplified fragment length polymorphism profiles of two procumbent and twining stem forms of *Basella*. Representative profiles of four primer combinations. *a*, E-ACT/M-CAT: I, E-ACT/M-CTA: II, E-ACT/M-CTC: III, E-ACT/M-CTG: IV. Unique DNA fragments (arrow marked) in twining stem with respect to two procumbent stems in following primer combinations: E-AAC/M-CAG: *b*; E-AAC/M-CAT: *c*; *d*, E-ACA/M-CAA: *e*; E-ACA/M-CAC: *f*; E-ACT/M-CAA: *g*; E-ACT/M-CAC: *h*; E-ACT/M-CAG: *i-k*; E-ACT/M-CAT: *l*; E-ACT/M-CTA: *m-p*; E-ACT/M-CTC: *q, r*; E-ACT/M-CTG: *s*. Six lanes of each gel portion represent two each of three materials in the sequence from left – procumbent (green), procumbent (red) and twining. DNA size marker has been shown in extreme left of (*a*).

while omission of spots of both high (65–75 kD size range) and low (<25 kD) molecular mass was noted (marked in Figure 4 *a* and *b*).

Using gravitropism, either in positive or negative direction, plants can effectively take up water and nutrients from the soil or absorb light energy from the atmosphere by expanding either roots or leaves respectively¹⁰. The experiments on plant gravitropism are gaining momentum

lately in the area of research on plant responses in zero or microgravity conditions and particularly in spaceflight experiments¹¹. Plant organs display helical growth movements known as circumnutation, which is a rotary movement of elongating plant organs, such as roots, stems and tendrils. The tip of a plant organ exhibits ellipses, circles, or pendulum-like movements that can alternate between clockwise and counterclockwise directions¹². These

movements help plant organs find suitable environmental cues. The amplitude, period and shape of the circumnutation depend on the plant species, the plant organs involved, and the developmental stage of growth. Circumnutation interacts with other types of movements, such as tropisms^{13,14}, but the mechanism of circumnutation remains still largely unclear¹⁰.

Climbing plants grasp a support by various means as observed in the vines and shoots that need to be anchored for support than in the shoots of nonclimbing plants^{13,15,16}. It is, therefore, thought that circumnutation provides the motive power for the winding response of climbing plants, but there is no direct evidence for the causal relationship between circumnutation and winding response. Thus, the detailed mechanisms explaining the relationships among graviresponse, circumnutation and winding response are still obscure. To unravel such a mystery of plant life form, one needs suitable variant or mutant. A gravitropic mutant of Japanese morning glory (*Pharbitis nil* or *Ipomoea nil*), Shidare-asagao (weeping), is defective not only in circumnutation but also in the

winding response, hence was characterized for this purpose¹⁰. This phenotype is similar to that of the *Arabidopsis* *SCARECROW* (*SCR*) mutant. However, more information from the repository of wild gene pool, and particularly from a non-model system like *Basella*, as the present study material would probably enhance the knowledge base about the phenomenon of circumnutation.

The twining or circumnulating stem form of *Basella*, revealed significant differences in terms of morphological characters in comparison to the two procumbent stem forms. A critical look at the morphological data between the two procumbent stem forms, however, did not result in any noteworthy differences, the only difference being the colouration. The difference between the procumbent and circumnulating stem forms was quite distinct particularly in terms of biomass since the circumnulating form showed reduced laminar area and weight. The large sized fleshy leaves of the procumbent stem forms, probably are the resultant of long human selection pressure particularly towards the direction of a useful leaf vegetable. The circumnulating form, on the other hand, has plausibly

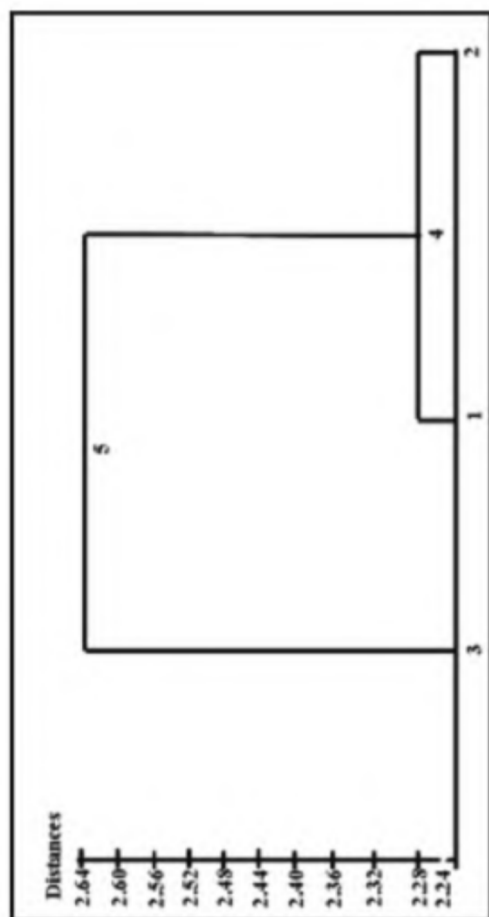


Figure 3. Dendrogram on the basis of results from AFLP analysis: 1–2 – procumbent (green), procumbent (red); 3 – twining *Basella*.

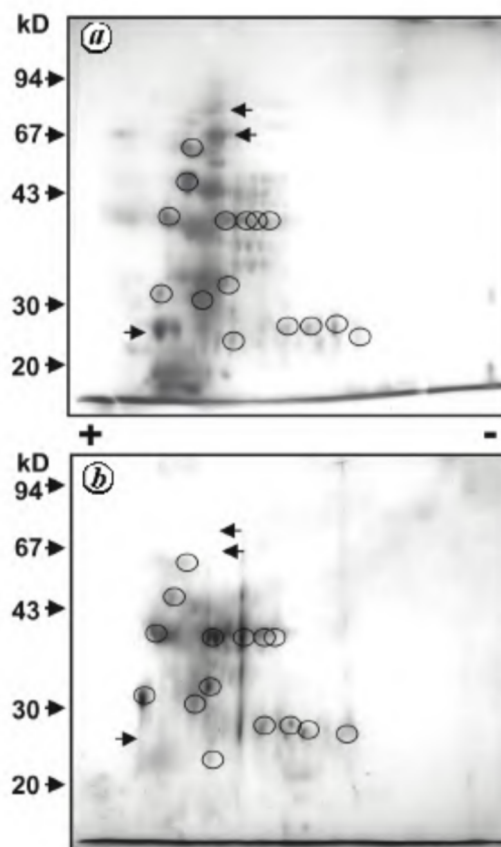


Figure 4. Two dimensional gel electrophoresis of total protein extracted from appropriate stem portions (procumbent/twining) of *Basella*: procumbent red stem (a) and twining stem (b). Distinct common spots have been indicated as circles and unique protein spots are arrow marked.

relocated its energy towards climbing or twining. This was clearly evident from its long internodes with very low stem circumference in comparison to the procumbent forms, which is particularly suitable for the purpose of twining around a support. Localization of numerous vascular bundles in a peripheral ring below the epidermis as revealed by stem anatomy further supports its adaptation for circumnutation. A 60% increase in length of the cells in the bending zone in the free-moving part of the shoot in *Phaseolus vulgaris*¹⁷ probably provides a circumstantial evidence of our present investigation.

No difference between the procumbent and twining stem forms in terms of cytology and pollen morphology probably validates the assumption that all the three materials under study of two stem forms, essentially belong to one single species, though they have been previously described cytologically or botanically under different species^{5,6}. The credential of the circumnuting stem form at least as a distinct variant, if not mutant, was probably reflected from the genomic and proteomic results. The distinction of the twining form from either of the two procumbent stem forms was nicely reflected in the AFLP profiles since not less than 18 and 11 unique DNA fragments were either omitted or appeared respectively in case the twining form with respect to both the procumbent forms. Results of AFLP analysis were aptly reflected in the constructed phenogram on the basis of per cent similarities among the three phenotypes under study, which showed a divergence of the twining or circumnuting form from the cluster of two procumbent forms.

The trend of data was again similar in the analysis of protein spots in two dimensional gel, where protein was extracted from appropriate stem portions (procumbent/twining) of respective matured plants to gain some insight into the protein profiles at a particular stage of circumnutation. Though there was gross similarity between the protein profiles of procumbent and twining forms, omission of some unique spots in the twining form at a particular developmental stage probably calls for further investigation on the characterization of these omitted proteins at the time of circumnutation in comparison to the procumbent one, which never showed any tendency towards climbing in spite of our efforts to provide this phenotype with solid support.

Considering the aforesaid results, it can be concluded that the circumnuting stem form has gained the specialized attribute of twining, the loss of certain unique DNA fragments in the genome or few proteins in the proteome, has probably contributed to this change in function; and this phenotype with all probability is a stable variant of the procumbent form of *Basella*.

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