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Research Paper

Genetic Analysis of Structure and Function of Stipules in Pea (*Pisum sativum*)

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To reveal the role(s) of stipules, pea genotypes of nearly common genetic background, but differing in the combination of wild type and mutant alleles of *STIPULE-REDUCED*, *AFILA* and *TENDRIL-LESS* genes together with *COCHLEATA* gene, were grown in a field in completely randomized design during the winter-spring seasons through 2006 to 2011 at the farm of the institute. The genotypes were characterized organ-wise, for morphological and anatomical features, photosynthesis and biomass accumulation and partitioning. Stipules were found similar to leaves/leaflets in respect of stomata shape, size and frequency as well as size and shape of pavement cells in epidermis and internally, in possession of palisade- and spongy-mesophyll parenchyma and veins in high density. In contrast to leaflets, which possess single primary vein and smooth lamina margin, the stem proximal part of stipule had serrations and possessed several primary veins; however, its distal part had single primary vein and smooth lamina margin. Simultaneous occurrence of the properties of simple and pinnately compound and palmate lamina structure were identified in stipules. Compared to leaflets in which the highest order of veinal reticulation was quinternary that in stipule was sexternary. Stipules were highly effective photosynthetic organ, responsible for about 30% of photosynthesis in phytomeres. Stipules enlarged plant's photosynthate/biomass production and accumulation capacity by about 72%. The presence of stipules avoided feedback inhibition of photoassimilation in leaves, by ensuring that pods/seeds remained the principal sink for photoassimilates. The presence of stipules was essential for high harvest index and grain yield; the harvest index in the stipule-deficient genotypes was 2.8 fold lower as compared to genotypes possessing wild type stipules. The plants in which the stipules were intact but leaves had been surgically removed, produced seeds proving that stipules alone were sufficient for survival and completion of life cycle in pea plant. The plants from which stipules had been surgically removed had reduced growth indicating that the presence of stipules was vital for continued shoot growth. The observations altogether demonstrated additive interaction between stipules and leaves in the accomplishment of photosynthesis, accumulation of biomass and the production of seeds in high yield with high harvest index. It was concluded that the presence of photosynthetically efficient stipules is a requirement in the breeding of high yielding cultivars of the pea (*Pisum sativum*) crop.

Key Words: Biomass Partitioning; Grain Legume; Harvest Index; Leaf and Stipule Genes; Pea Leaf; Photosynthetic Sink; Stipule Morphology and Anatomy; Stipule Photosynthesis

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1. Introduction

Deciduous or persistent stipules, formed as rudimentary or prominent bifacial leaf-like organs, spines or tendrils, attached to stem in pairs per phytomer, are known to occur in many species of plants. When present, their morphology has often served as a taxonomic trait. The functions hitherto ascribed to stipules are speculative. Chlorophyllous stipules have been assumed to contribute to the photosynthetic potential of their bearers. Stipules of various morphologies are thought to improve the adaptiveness of stipulate species, including protection against pests. The paplionoid fabaceae field pea annual crop plant *Pisum sativum* (2n = 14; ≥ 5000 Mbp nuclear genome), which is a useful model for genetical investigations on leaf, inflorescence and flower morphogeneses, is a stipulate plant. The genetic material presently available in this system allows dissection of functions performed by stipules.

Each node of *P. sativum* plant bears a persistent large laminate simple peltate stipule on either side of the junction of leaf petiole with stem [1]. The leaf has compound leafblade comprising up to 15 pinnae. The leafblade rachis, extension of the petiole, bears on it up to 3 pairs of simple leaflets on the petiole proximal side (proximal domain of leafblade), up to 4 pairs of simple tendrils on the side distal to petiole (distal domain) and an apical/terminal simple tendril. A variety of natural and induced mutant forms of stipules and leaves are known in *P. sativum*. In the plants mutated in the *STIPULE-REDUCED (ST)* gene [2], the nodes bear a pair of sessile and small laminated stipules of knife-blade-like morphology. Hyper-variation is observed in the stipule composition at different nodes in the plants mutated in the *COCHLEATA (COCH)* gene [3-7]. The *coch* mutant stipules can be simple or compound. The compound *coch* stipules have leafblade-like morphology. The simple *coch* stipules may be sessile or petiolated, hairy or spatulately laminated. A node of *coch* mutant can have nil, one or two stipules; the structure of the stipules, when two are present at a node, may be different. A large majority of nodes are barren of stipules in the *coch st* double mutant plants. The plants mutated in one or more of the following major genes are known to bear leaves of abnormal and different (mutation-dependent) morphologies- *UNIFOLIATA* (*UNI*), *STAMINA PISTILLOIDA*, *INSECATUS*, *CRISPA, MULTIFOLIATE-PINNA*, *AFILA* (*AF*) and *TENDRIL-LESS* (*TL*); the morphology of their stipules is normal [5, 8-27]. For example, the leaves of *uni* mutant plants are simple or lobed and not compound. In the *af* mutant plants, the distal and terminal tendrils are simple but the proximal leaflets are replaced by compound tendrils, thereby the compoundness of proximal domain gets increased. All pinnae are simple leaflets in the *tl* mutant. The lines of pea, of a common genetic background, in which wild type and mutant alleles of the *coch* and *st* stipule mutations and *af* and *tl* leaf mutations have been recombined in various combinations are now available for studying the interactive effects of stipules and leaves, on the features of plant growth and development.

In the plants of vegetative phase, stem, stipules, petioles, rachis, leaflets and tendrils have chlorophyllous green colour. At the stage of flowering-cum-fruiting, the chlorophyllous green colour is shared by some additional plant structures/ organs, including inflorescence pedicel, floral calyx and carpels, pods and developing embryos. Surface-area-wise, stipules are the major chlorophyllus organs, next to leaves. Being chlorophyllus, it is believed that stipules are photosynthetic. However, the relative contribution of stipules versus leaves to photosynthesis at nodes is not yet quantitatively characterized [28, 29]. In analogy of the dependence of plant's vegetative and reproductive growth on leaf photosynthesis [30, 31], the relative effects of stipule and leaf photosynthesis on the growth properties of pea plant are also not fully understood [29].

In this study, the hypothesis that stipules in the pea crop plant are leaf-like and are essential for obtaining high rates of photosynthesis and optimization of biomass accumulation for high harvest index is evaluated, by comparing some of the concerned properties between the wild type and the mutant lines, harboring stipule and leaf mutations in various combinations, with similar genetic background. In particular, the following questions

have been addressed: (a) are stipules essential for plant to complete its life cycle?; (b) can stipules, in the absence of leaves, support plant's life cycle?; (c) is the lamina anatomy of stipule and leaflet congruent?; (d) what is the contribution of stipules to the total phytomere photosynthesis?; (e) do stipules improve plant growth?; and (f) how is harvest index affected by stipules? The study shows that the stipule, is complementary to the leaf for life cycle attainment, is anatomically similar to the leaflet/leaf, adds to photosynthetic potential, biomass accumulation and vegetative and reproductive growth, and is essential for high harvest index.

2. Material and Methods

(a) Plant Genotypes

The mutant alleles of *COCH*, *ST*, *AF* and *TL* genes used in the study are from the Blixt collection [32]. The crossing programmes used to construct the genotypes included in the study have been described earlier [7]. As per their pedigree, the background of all the genotypes was homogenized by one, two or three backcrossings with the SKP-1 line. Phenotypes of the wild type, *coch*, *st* and *coch st* stipules and wild type, *af* and *tl* leaves have been mentioned in the introduction section. The stipules and leafblade phenotypes of all the 12 genotypes included in the study are shown in Fig. 1.

(b) Growth Conditions

Pea crops for the study were raised in an experimental field of the Institute at New Delhi (28° 35´N; 77° 12´E), in the winter/*rabi* (October to March) seasons of 2006 to 2011. Genotype-wise 10 seeds were sown in 1m long row, and the row to row distance was kept 75 cm. For each experiment, the genotype rows were replicated five times (or as mentioned otherwise) and all the rows were completely randomized. The soil of the plots used was sand loam. To prepare them, the field plots were solarized, ploughed, irrigated, applied N, P and K fertilizers at 60-80, 50 and 50 kg/ha rates, harrowed and levelled. The crops were irrigated 4 times or as and when required. The crops were applied one spray each of 0.1% chloropyrophos and diethane M45 to prevent insect pests and fungal infection, at the time of onset of flowering.

(c) Photosynthetic Measurements

The crops of six genotypes were grown for this experiment in 2006-07, 2007-08 and 2008-2009 winter seasons. The 2006-07 and 2008-09 experiments were used to standardize the methodology and confirm the extreme results, respectively. Photosynthesis was measured at three developmental stages: at 50 days from germination when the crop was still in its vegetative state, at 80 days when flowering had set in and at 100 days when plants bore mature pods. A day prior to each of the photosynthesis sampling time, the crop was irrigated to keep the soil fully hydrated. One randomly selected plant per genotype was sampled per replication at each stage of development. On the plants selected for photosynthetic measurements, stipules and leaves were sampled from three consecutive nodes, 5 nodes below the apex. The gas exchange analysis was recorded using GFS-3000 portable gas exchange fluorescence system (Heinz-Walz, Effeltrich, Germany). Intact individual stipules and pinnae, rachis and petiole segments from each of the leafblade domains were used to analyze the gas exchange. All the photosynthetic measurements were recorded between 10 AM and 2 PM, at light intensity around 1000-1200 $\mu mol.m^{-2}.s^{-1}$ and CO_2 concentration around 380 ppm. After the gas exchange analysis, the leaf part used was harvested, labeled and placed in folds of a blotting paper overnight. The leaf organs were traced on mm graph paper to estimate their areas. Net photosynthesis recorded by the instrument in µmol of CO_2 utilized per meter square of the leaf area per second ($\mu mol.m^{-2}.s^{-1}$) was multiplied by the total area of the leaf part to get the total photosynthesis as µmol of CO_2 utilized per second ($\mu mol.s^{-1}$). The whole leaf photosynthesis was calculated by multiplying the total photosynthesis of the leaf parts with the total area of each leaf. For each photosynthetic parameter, variation between replications of a genotype was estimated from means of measurements at the three nodes of the sampled plant(s) at the three developmental stages.

(d) Estimation of Dorsal Surface Area and Biomass of Leaf and Stipules

These measurements were made for the genotypes

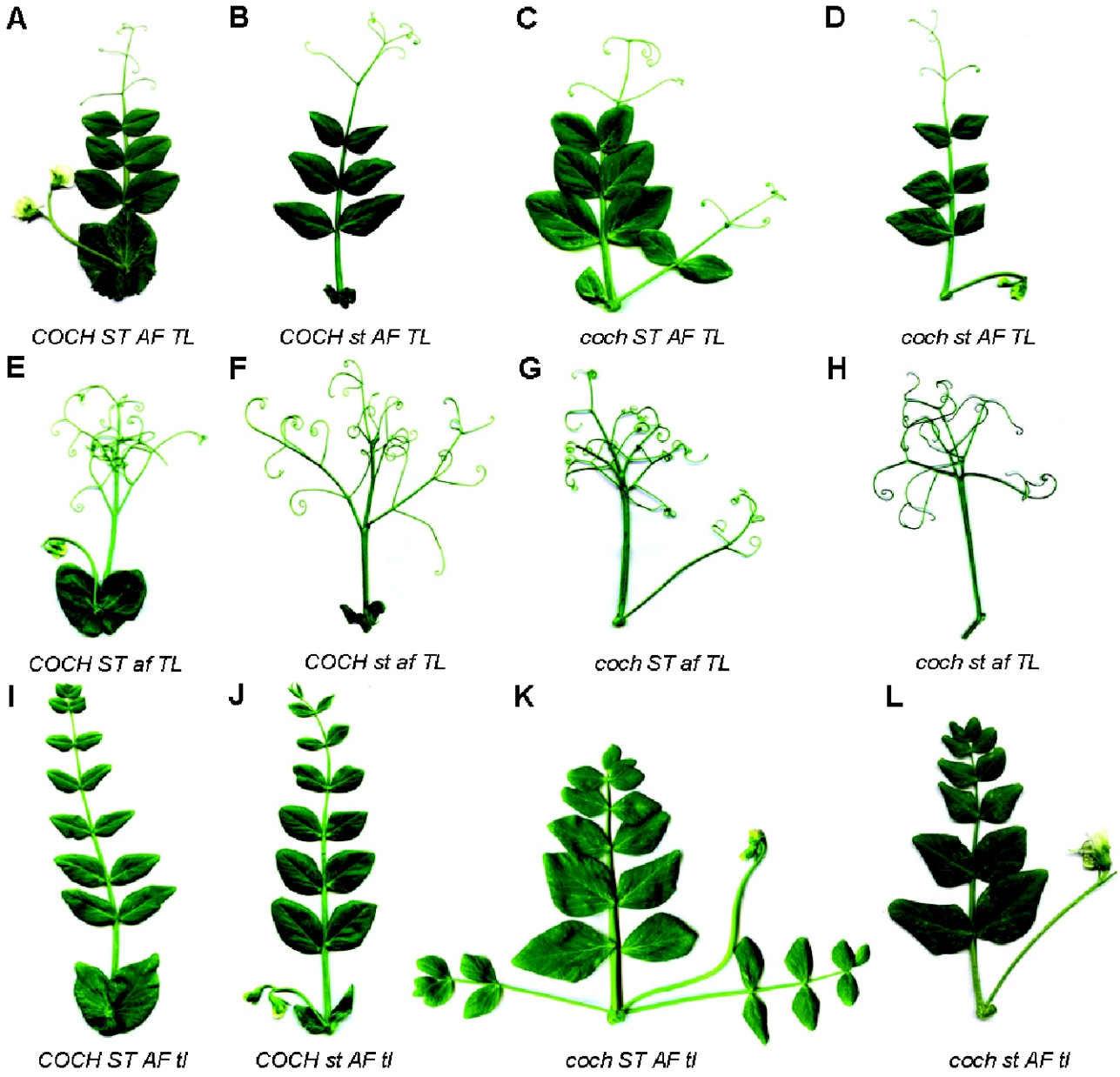

Fig. 1: Morphologies of the stipules and leaf, borne on the first flowering node or node(s) immediately below it, in the 12 genotypes of *Pisum sativum* deployed in this study

included in the study of photosynthesis. One plant per genotype per replication was randomly selected for these assays. The stipules and leaves were cut out from the first flowering node and fifth node below it and fifth node above it as samples, from the selected plants. The boundaries of the samples were traced on mm graph paper for the estimation of their areas. The same samples were subsequently dried and weighed for the biomass estimations.

(e) Anatomical Studies

The stipules and leaves of six genotypes examined for photosynthesis were studied anatomically. The samples were taken from the crops of genotypes grown in the 2009-10 season. For the study of their surfaces and venation patterns, leaves/leaflets/tendrils and stipules were cleared. The organs were fixed overnight in 70% alcohol, transferred to phenol:

lactic acid : glycerol : water :: 1 : 1 : 1 : 1 mixture and cleared by incubation at 90° C for 15 min. The cleared organs were stored in 20% glycerol. The organs were examined microscopically by staining with dilute safranine (as provided by the manufacturer Qualigen), washing in 5% alcohol and mounting on slides in 20% glycerol. The observations were recorded and photographs were taken at 40X, 100X and 400X magnifications, using Nikon E100 microscope and Nikon 8400 digital camera. To estimate the densities and sizes of stomata, pavement cells and veins, the pictures were printed on mm graph papers. The vein lengths were measured by superimposing thread on each primary, secondary, tertiary or higher level vein in an area which magnified $1mm^2$ and then a scale was used to measure the thread length that covered a vein length. The number of branch points and free veins were also counted from magnified pictures.

The adaxial-abaxial lamina or tendril thickness and sizes of different types of tissues and cells were estimated from the transverse sections of stipules, leaflets and tendrils. The sections were cut with hand held razor. The material to be cut was supported between splits of radish. The sections were stained with dilute safranine and mounted in dilute glycerine for microscopic examination and photography, as described above.

(f) Estimation of Seed Harvest Index in Leafblade Minus Plants

Ten rows of *COCH ST af* were sown, in the winter 2006-07 season. Five plants were labeled in each row. Such plants in the alternate rows were treated differently. The treatment consisted of surgical removal of leafblades from fifth node onwards. To remove leafblade, an incision was made on petiole as soon as it was out of the fold of stipules. In the control plants, leafblades were not incised. In the course of this experiment, one or two labeled plants dried prematurely in the two rows of the surgical treatment. The experimental plants were uprooted and pooled treatment- and row- wise, dried first at 80° C for 2h and later at 37° C and weighed. Subsequently, pods were shelled to obtain the weight of seeds. The observations on the surviving experimental plants were used to estimate the average biomass distribution among the vegetative parts and seeds in single plants of the two treatments.

(g) Estimation of Plant Growth in Stipule Minus Plant

Two rows of a *COCH ST af* genotype were grown. Five plants in a row were given a surgical treatment and plants in the other row served as control. In the treated plants, stipules were removed soon after their appearance, from sixth node onwards. The growth was compared in terms of the number of nodes formed in the two set of plants.

(h) Organ-wise Biomass Assessments

This experiment was accomplished using 12 genotypes in the 2007-08 and 2008-09 seasons (Table 7). Three plants per genotype per replication, aged 14 weeks, were sampled two weeks before the normal harvest time. Entire uprooted plants were pooled genotype- and replication-wise. Pods, stipules, leaves, stem and root were separated and dried first at 80° C for 2h and later at 37° C. Roots were carefully washed to remove the soil before their incubation for drying. All the dried organs were weighed separately. Later pods were shelled to record the weight of seeds. The data were statistically analyzed to arrive at the genotype-wise estimates of mean and standard errors and to test the difference between individual and sets of genotypes critical difference estimates were made. These observations were also used to estimate Pearson's coefficient of correlation between the estimates of some of the parameters.

(i) Assessment of Stipule Occupancy and Fertility

These observations were recorded in the 2008-09 season. Five plants/genotype were sampled at the time of biomass assessments. The number of stipules formed on the twenty nodes and the total number of seeds formed in all the pods were counted plant-wise.

(j) Statistical Analysis

The data were subjected to analysis of variance for completely randomized design. Standard errors were calculated to indicate variability for each of the estimated means. Critical difference (CD) values were calculated for making inter-genotype comparisons.

Correlation between variables was determined using the Pearson's coefficient. Each statistical analysis was carried out using the SPSS 16.0 software programme for windows.

3. Results

(a) Sustenance of Growth and Reproduction by Stipules

Among the genotypes of pea studied here, the *af* mutants have leafblades with the least surface area. Since many *COCH ST af* genotypes are in commercial cultivation, the high seed harvest index in their plants means occurrence of photoassimilation, biomass accumulation and biomass partitioning to seeds at high levels. However, the reproductive behaviour of these pea plants that possess stipules but lack leaves remains unknown. This question was examined in the plants of *COCH ST af* genotypes, by surgical removal of leafblades from all the non-embryonic nodes while stipules were kept intact. The plants from which leaves had been surgically removed completed their life cycle, formed pods and produced seeds, albeit with low harvest index (Table 1). These results demonstrated the significance of stipules in the absence of leaves, in the sustenance of plant survival, growth and reproduction. These observations indicate that stipules serve as major providers of photosynthetic assimilates.

(b) Retarded Plant Growth in Stipule Minus Plant

The effect of presence of stipules on plant growth was investigated in a *COCH ST af* genotypes. In a set of plants, the stipules were surgically removed as they appeared from the sixth node onwards on the primary shoot. In the control plants, the stipules were allowed to remain intact on the host plants. The plant growth was compared between the two sets of plants at the time of the onset of flowering. Stipules removal had an adverse effect on the plant growth. Whereas stipule bearing plant produced 48 nodes on an average, of which 14 were on the main branch, the stipule deficient plants produced in all 30 nodes and only 11 nodes on the main branch. The surgical removal of the stipules had two effects: (a) premature arrest of the growth on the main branch and (b) deficiency in the production of secondary branches (Fig. 2).

(c) Survival in Stipule Deficient Genotypes

It has been possible to construct homozygous lines in which *coch* and *st* mutations are simultaneously combined with leaf morphology mutations *af*, *tl*, *mfp* and *uni-tac* in all possible permutations [7, 33 and unpublished work]. A common feature observed in plants of these 16 *coch st* lines (having variable genetic background) is absence of one or both stipules from large majority of nodes. Stipule occupancy varied from 16 to 39% in these *coch st* lines as compared to 100% in the wild type. Under natural field conditions each one of the 16 *coch st* lines produced some fertile seeds (0 to several seeds/plant), despite that their sexual fertility was highly compromised. The stipule occupancy in the first twenty nodes and the number of grains produced on the whole plant in the *COCH ST AF TL*, *coch st AF TL*, *coch st af TL* and *coch st AF tl* lines of constant genetic background were 40 ± 0 and 55.4 ± 2.2, 9.0 ± 0.7 and 16.0 ± 1.4, 14.6 ± 2.3 and 3.0 ± 0.7, and 7.0 ± 1.4 and 29.4 ± 1.6, respectively. These observations show that the presence of stipules is not essential for

Table 1: Effect of surgical removal of leafblade, from a large majority of nodes, on the biomass accumulation and harvest index, in the plants of *COCH ST af* genotype

Parameter[a]	Leafblade plus plant (mean±S.E.M.)	Leafblade minus plant[b] (mean±S.E.M.)
Dry weight of vegetative parts (g,A)	4.9±0.23	1.9±0.24
Dry weight of seeds (g,B)	2.3±0.22	0.5±0.29
Harvest index [C=(B/A+B)×100]	32.3	19.1

a, n = 25;

b, The seeds produced fertile plants of *COCH ST af* morphology which bred true to genotype.

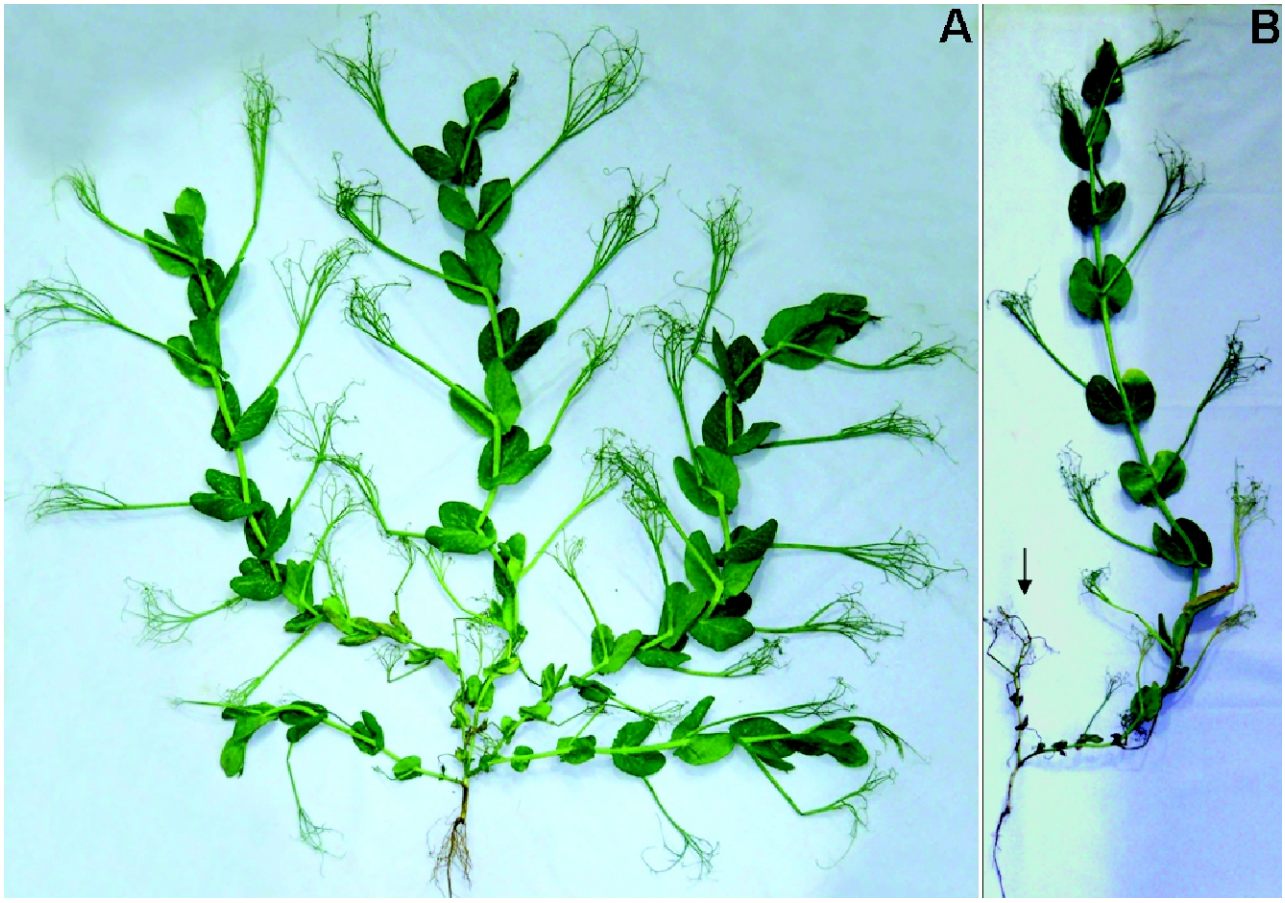

Fig. 2: Effect of surgical removal of stipules on the growth of *COCH ST af* plants: (A) A plant on which stipules were kept intact. (B) A plant from which stipules had been surgically removed from sixth node onwards, on the primary shoot. The arrow in B indicates the primary shoot from which stipules had been removed from non-embryonic nodes

the survival of pea plant; *coch st* stipule-deficient genotypes are competent in producing progeny.

(d) Photosynthesis in Stipules and Other Plant Organs

The rate of photosynthesis (µmol $CO_2.m^{-2}.s^{-1}$) and net photosynthesis (µmol.s^{-1}) were measured in stipules and leaves for several phytomeres, at the vegetative, flowering and pod maturation stages of plant growth, in *ST AF TL*, *st AF TL*, *ST af TL*, *ST AF tl*, *st af TL* and *st AF tl* genotypes of a common genetic background. All of the observations (Table 2) considered together showed that in the pea plant, stipules comprise a major photosynthetic component of phytomeres. Although stipules accounted for only about 29% of the total photosynthesis of a node, the rate of photosynthesis was about 82% higher in stipules than in leaves.

The stipule morphology mutation *st* was observed to improve the rate of photosynthesis in stipules, without significantly affecting photosynthesis in leaves (leaflets/tendrils, petiole and rachis; Table 2). The stipule rate of photosynthesis was about 90% higher in *st* genotypes than in *ST* genotypes. Photosynthesis was about 80% less in *st* stipules than in *ST* stipules because of the latter's larger size. In *st* genotypes, bulk of photosynthesis (~ 0.85) at a node occurred in leaf. The rate of photosynthesis in stipule was decreased significantly but variably by the leafblade morphology mutations *af* and *tl* (Table 2). Stipule's rate of photosynthesis in *tl* genotypes was about 32% lower than in *TL* genotypes. However, the *tl* mutation increased the nodal photosynthesis by about 90% (Table 2) via increase in the surface areas of the component organs - stipules and leaf. Stipules accounted for about 0.35

Table 2: Rate (µmol.m^{-2}.s^{-1}) and net assimilation (µmol.s^{-1}.10^{-4}) of CO_2 in stipules and leaves of *ST AF TL*, *st AF TL*, *ST af TL*, *ST AF tl*, *st af TL* and *st AF tl* genotypes of pea *Pisum sativum*†

Genotype			Rate of photosynthesis (mean±S.E.M.)		Net photosynthesis in (mean±S.E.M.)			% net photosynthesis in (mean±S.E.M.)	
ST	*AF*	*TL*	Stipule	Leaf	Stipule (S)	Leaf (L)	S+L	S	L
+	+	+	10.1±0.97	12.3±0.58	247.0 ± 10.69	444.3±29.49	691.3±25.76	35.7±2.16	64.0±2.16
+	+	-	8.8±0.22	7.7±0.65	310.3±8.67	459.0±27.15	769.3±24.01	40.3±1.85	59.7±1.84
+	-	+	11.2±0.85	5.2±0.70	104.3±11.98	108.0±11.27	212.3±22.24	49.1±0.04	50.9±0.04
-	+	+	29.4±0.77	10.8±1.24	35.7±2.33	340.7±23.67	376.3±22.88	9.5±0.92	90.5±0.92
-	+	-	13.4±0.51	8.7±0.92	94.3±5.93	463.0±26.85	557.3±32.63	16.9±0.22	83.1±0.22
-	-	+	14.2±0.79	3.1±0.78	23.7±7.54	91.0±17.61	114.7±10.11	22.1±8.53	77.9±8.53
$F_{5:12}$			107.8**	16.6**	200.8**	55.2**	121.2**	16.9**	16.9**
CD*‡			2.3	2.6	25.4	72.6	73.7	11.4	11.4
CD**			3.2	3.7	35.7	101.9	103.4	16.0	16.0
Range			8.8-29.4	3.1-12.3	23.7-310.3	91.0-463.0	114.7-769.3	9.5-49.1	50.9-90.5
Mean of all genotypes			15±1.8	8±0.8	136±27.5	318±41.2	454±62.0	29±3.6	71±3.6
ST genotypes			10±0.4	8±1.2	221±35.1	337±66.2	558±100.5	42±2.3	58±2.2
st genotypes			19±3.0	8±1.3	51±12.6	298±63.1	349±64.3	16±1.9	84±1.9
$F_{1:12}$			227.5**	1.7	633.7**	4.1	113.9**	72.2**	72.1**
TL genotypes			16±2.6	8±1.3	103±29.6	246±50.4	349±72.9	29±5.0	71±5.0
tl genotypes			11±1.4	8±0.4	202±62.4	461±1.2	663±61.2	29±6.8	71±6.8
$F_{1:12}$			66.2**	0.2	195.2**	111.2**	231.3**	0.02	0.02
AF genotypes			15±2.8	10±0.6	172±37.0	427±16.7	599±49.7	26±4.3	74±4.3
af genotypes			13±0.9	4±0.7	64±23.3	100±4.1	164±28.2	35±8.2	65±8.2
$F_{1:12}$			18.6**	61.7**	228.4**	257.6**	442.2**	9.7**	9.7**
$F_{1:12}$ (af vs tl)			4.9*	23.2**	282.0**	235.8**	437.7**	3.6	3.6

*, ** = F values from analysis of variance following the completely randomized design, with degrees of freedom given as subscript: * F significant at 5 % level, ** F significant at 1% level; † = Each estimate is average of observations on 3 phytomeres × 3 developmental stages × 3 replications; ‡ = critical difference values, * at 5% and **, 1% level.

of the photosynthesis at a node in *af* genotypes, which was significantly higher than the corresponding estimate of 0.26 for the *AF* genotypes (Table 2). The nodal photosynthesis (stipules + leaf) in *af* genotypes was about 4 fold lower than *AF* genotypes, although the rate of photosynthesis was significantly (about 15%) higher in *af* genotypes than in *AF* genotypes (Table 2).

On account of the hetero-stipulation in *coch* genotypes and the absence of stipules from majority of nodes in *coch st* genotypes, complementary study of photosynthesis in stipules of these genotypes was not feasible. The stipule when present in *coch* and *coch st* genotypes was either simple, similar in morphology to leaflet with large petiolule and very

small in size to large like most proximal leaflets, or compound like leaf but comparatively smaller in size. The rate of photosynthesis (μ mol CO_2 assimilated per m^2 per sec) was estimated in simple and compound stipules borne on *coch ST AF TL* plants. It was 2.8 ± 0.1 and 9.1 ± 0.3, respectively, for simple stipules of size ≤ 25 mm^2 and ≥ 100 mm^2 and 7.5 ± 0.8 for the compound leaf-like stipules. The photosynthesis rates in large simple stipules and compound stipules of *coch ST AF TL* were roughly similar in magnitude to the mean photosynthesis rates for leaflets and compound leaf of all the 6 genotypes (Table 2), respectively.

The rate of photosynthesis at the internodes of phytomers, sampled for stipular and leaf photosynthesis from *COCH ST AF TL* plants, was 1.5 ± 0.4, lower than that of petiole, rachis and tendrils of the leaf. The contribution of pods to photoassimilation was negligible. The rate measurements among concurrently borne pods of different stages of development varied between –7.4 and +2.6.

(e) Negative Relationship of Photosynthetic Rate with Organ Specific Weight

The photosynthetic rate was observed to be higher in genotypes that bore stipules and leafleted leaves of thinner lamina. On the basis of surface area and biomass measurements of stipules and leaves (Fig. 3) and biomass measurements of the organs (Table 7), the genotypes, among those examined for photosynthesis and whose leaves possessed leaflets, fell in the following order in terms of specific weights of stipules and leaves: *st AF TL* < *ST AF TL* < *ST AF tl*. Interestingly, the order of the genotypes in terms of the photosynthetic rate of stipules and leaf was antiparallel: *st AF TL* > *ST AF TL* > *ST AF tl*. Lower specific weight of organ meant its lamina must be thinner. The examination of transverse sections of the stipules, leaflets and tendrils borne on various genotypes showed that the laminae or tendrils were thinner in stipules or leafblades that had

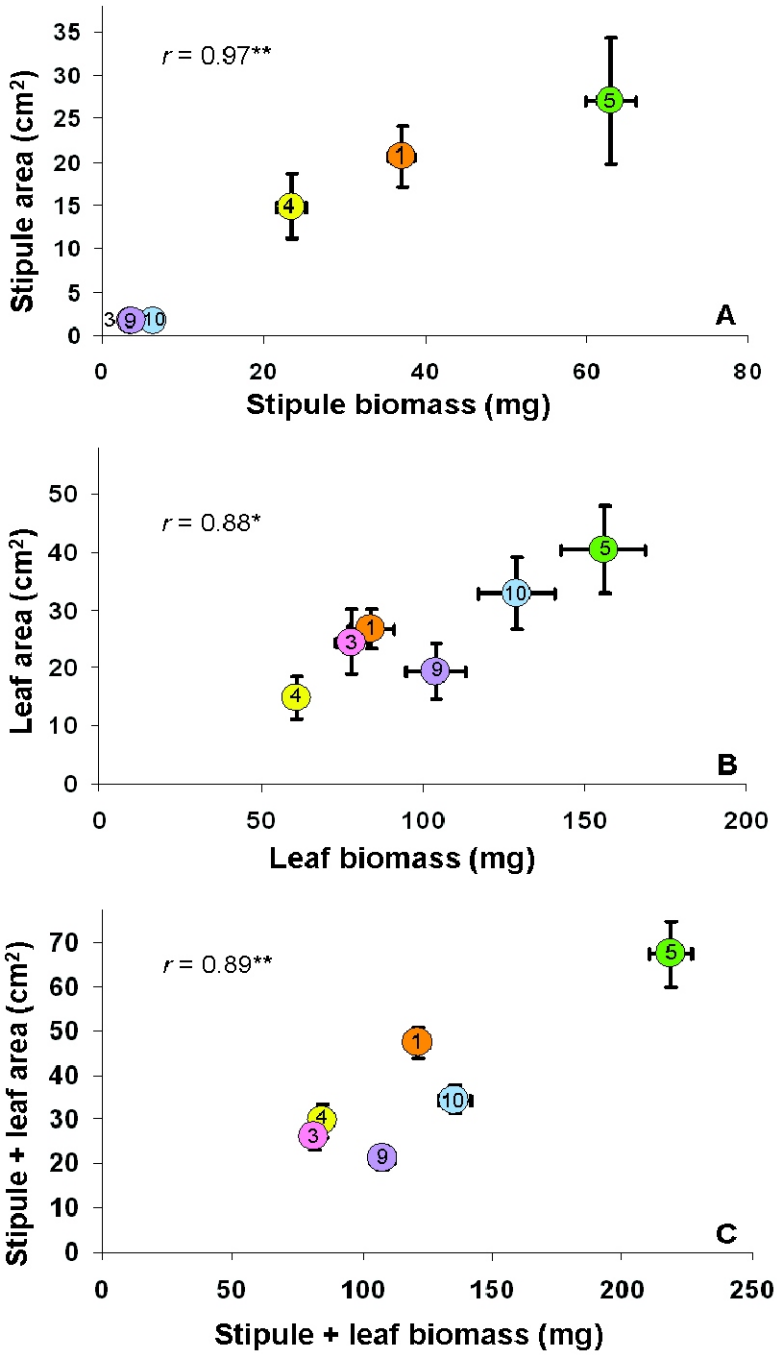

Fig. 3: Relationships between biomass and dorsal surface area of stipules (A), leaf (B) and stipules + leaf (C) in the *ST AF TL* (1), *st AF TL* (3), *ST af TL* (4), *ST AF tl* (5), *st af TL* (9) and *st AF tl* (10) genotypes of pea *Pisum sativum*. The genotype numbers given in parenthesis conform to those given in the Table 7. The estimated values of Pearson's coefficients of correlation (*r*) between biomass and area for stipules, leaf and stipules + leaf were positive and highly significant

Table 3. Anatomical features of stipules, leaflets and/or tendrils, borne on the *ST AF TL*, *st AF TL*, *ST AF tl* and *ST af TL* genotypes of pea *Pisum sativum*, revealed by transverse sectioning of the organs

Genotype	Organ	Adaxial-abaxial thickness† (mean±S.E.M.) (μm) of		Area of midrib vascular bundle (mean±S.E.M.) ($\mu m^2 \times 10^2$)	Palisade mesophyll parenchyma			Spongy mesophyll parenchyma	
		Midrib region	Flat lamina sub-median region		Present(P) / Absent(A)	Transectional cell area (mean±S.E.M.) (μm^2)	P/A	Transectional cell area (mean±S.E.M.) (μm^2)	No. of layers (mean±S.E.M.)
ST AF TL	Stipule	593±24.4	243±25.1	236±16	P	744±188.9	P	629±272.2	5±0.5
st AF TL	Stipule	427±16.7	152±10.3	96±3	P	445±71.3	P	438±151.4	5±0.6
ST AF TL	Leaflet	646±48.3	256±31.7	256±33	P	771±177.4	P	421±144.4	6±0.6
ST AF tl	Leaflet	715±18.4	272±23.2	321±9	P	904±49.8	P	416±34.7	7±0.5
ST AF TL	Tendril	675±5.7	NA‡	NA	A	NA	P§	489±156.1	3±0
ST af TL	Tendril	773±87.0	NA	NA	A	NA	P§	320±24.2	4±0
F value		$F_{5, 12} = 23^{**}$	$F_{3, 8} = 15^{**}$	$F_{3, 8} = 74^{**}$		$F_{3, 8} = 6^{*}$		$F_{5, 12} = 1$	$F_{5, 12} = 26.4^{**}$
CD$^{\delta *}$		77	45	36		258		275	0.8
CD**		107	66	52		375		285	1.2

*, ** = F values from analysis of variance following the completely randomized design: * F significant at 5 % level, ** F significant at 1% level; † = diameter in case of tendril; ‡ = NA, not applicable; § = the cells below the epidermis that were morphologically similar to spongy mesophyll parenchyma cells and took stain similar in intensity to spongy cells; δ = critical difference values, * at 5% and **, 1% level.

higher photosynthetic rates (Tables 2 and 3; Fig. 3 and Fig. 4). For example, stipules of *st AF TL* had the highest photosynthetic rate and possessed thinnest lamina. On the contrary, among the leafleted leaf bearing genotypes, leaves of *ST AF tl* had the lowest rate of photosynthesis and leaflets had the thickest lamina (Table 3, Fig. 4). Thinness of the stipules of *st AF TL* genotype was correlated with smaller size of palisade- and spongy- mesophyll parenchyma cells and lower number of spongy parenchyma cell layers. The corresponding parameters in the leaflets of *ST AF tl* were the maximum observed (Table 3, Fig. 4). While the coefficient of correlation between the abaxial-adaxial thickness and the number of spongy parenchyma cell layers in stipules and leaflets was positive and highly significant, those between photosynthetic rate on the one hand and adaxial-abaxial thickness or number of spongy mesophyll parenchyma cell layers on the other hand were negative and significant (Table 4). The high rate of photosynthesis observed in *st* stipules as compared to *ST* stipules might be a consequence of

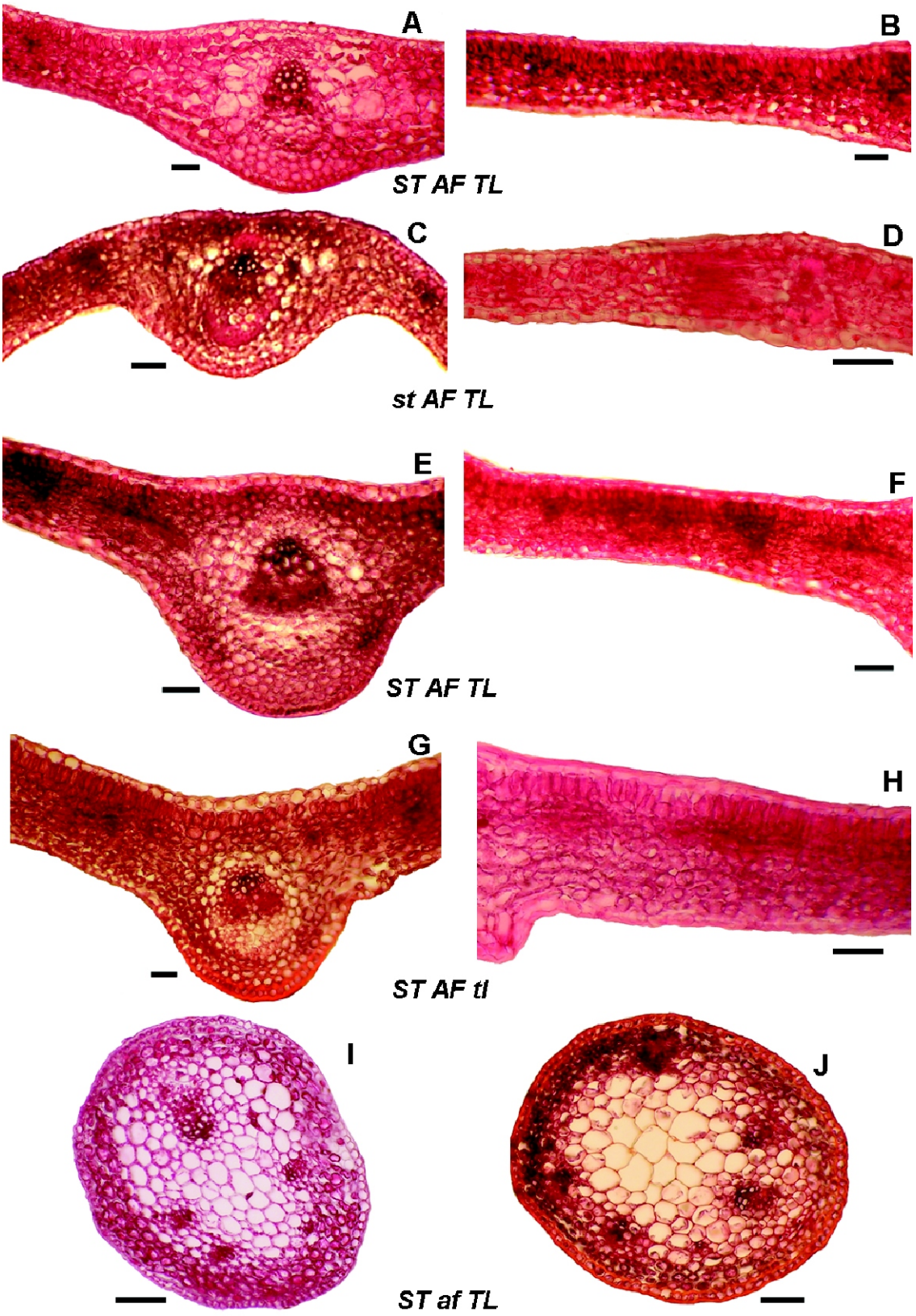

Fig. 4: Transverse sections of stipules, leaflets and tendrils of *ST AF TL*, *st AF TL*, *ST AF tl* and *ST af TL* genotypes, in the median (A, C, E and G)- and submedian (B, D, F and H)- area of stipules (A-D) and leaflets (E-H) and petiole proximal part of primary tendril (I and J). Scale bar = 100µm

Table 4: Pearson's coefficients of correlation (*r*) between photosynthesis rate (µmol CO_2 assimilated.$m^{-2}.s^{-1}$), abaxial-adaxial thickness (µm) and number of layers of spongy mesophyll parenchyma cells in the laminated organs, stipules and leaves, in pea *Pisum sativum*

Parameter	Photosynthetic rate	Adaxial-abaxial thickness
Adaxial-abaxial thickness	-0.85**	
Number of spongy layers	-0.62*	0.80**

*, ** = significant at 5% and 1% probability level, respectively.

higher density of chloroplasts in them as the size of *st* mesophyll cells was relatively smaller than that in *ST* stipules.

(f) Stipule Morphology and Anatomy

Morphological features of COCH ST, COCH st, coch ST and coch st stipules have been described earlier [2-7]. In the context of the present study, some of the characteristics of COCH ST stipules are mentioned. The stipule is sessile and contoured roughly like a human ear. Lamina on the two sides of midvein is asymmetrical. The leaf facing side has smooth margin. The opposite side is lobed and has serrated margin in the region proximal to stem and the distal part has smooth margin. The two stipules at a node are arranged as mirror images (antiparallely) of each other. Their margins overlap such that together they appear peltately arranged sheath around their phytomere stem at the node (Fig. 1A, E and I and Fig. 6A).

Shape and size of the epidermal cells, and size and frequency of the stomata inlaid in the epidermal layer were characterized for stipules, leaflets (of leaf) and tendrils borne on *ST AF TL*, *st AF TL*, *ST AF tl* and *ST af TL* genotypes (Fig. 5; Table 5). In the *ST AF TL* genotype, the pavement/epidermal cells of stipules and leaflets had similarly irregular cell wall boundary and were of about the same size (area $\simeq$ 2000µm^2; Fig. 5A and 5C). Stipules and leaflets bore stomata of about the same size ($\simeq$ 425µm^2). The frequency of the stomata on the epidermal layer of stipules and leaflets was also similar ($\simeq$ 180/mm^2). However, the properties of the epidermis of *ST AF TL* tendrils differed considerably from those of the stipules and leaflets of the same genotype. The boundary of the epidermal cells of tendrils was rectangular. The regular boundary meant lower surface/volume ratio in comparison to high surface/volume ratio of the cells with irregular boundary. The size of tendrillar epidermal cells was about 100 and 85% larger than that of the corresponding cells of stipules and leaflets. The frequency of stomata in tendrillar epidermis (85/mm^2) was significantly lower, about half of that in stipules and leaflets, although stomata of tendrils were marginally bigger.

The *st* mutation did not affect the frequency and size of stomata on epidermis. However, *st* mutation decreased the size of the epidermal cells by about 30%. The boundaries of the epidermal/pavement cells of *st* stipules were less irregular than that of the pavement cells of *ST* stipules. The *af* mutation was observed to have increased the size of the epidermal cells by about 45% without affecting much change in the size or frequency of stomata.

The leaflets in *ST AF TL* and *ST AF tl* genotypes had single primary vein, many secondary veins that arose pinnately from the primary vein and a few to several intersecondary veins (Fig. 6D and 6F). The arrangement of the major veins was somewhat different in stipules. In the stipules of *ST AF TL* plants (Fig. 6A; Table 5), there was a major primary vein from which secondary veins arose like in leaflets. Besides, there were several to many veins, smaller and minor in thickness, which also appeared to be primary but were concentrated in the stem proximal lobed and serrated expansion of the stipule lamina which sheathed the stem node. The origin of secondary veins from the supernumerary minor veins was pinnate. The supernumerary primary veins were also present in *st* stipules (Fig. 6B and C and 7); these stipules were lanceolate and did not sheath the stem. The number of primary veins was more than one in *AF* and *af* tendrils (Fig. 6E and 7M-P). The stipules shared the property of multiple first order veins with tendrils on the one hand and palmate type of leaves on the other hand.

In the *ST AF TL* plants, the highest vein orders observed in the stipules and leaflets were sexternary

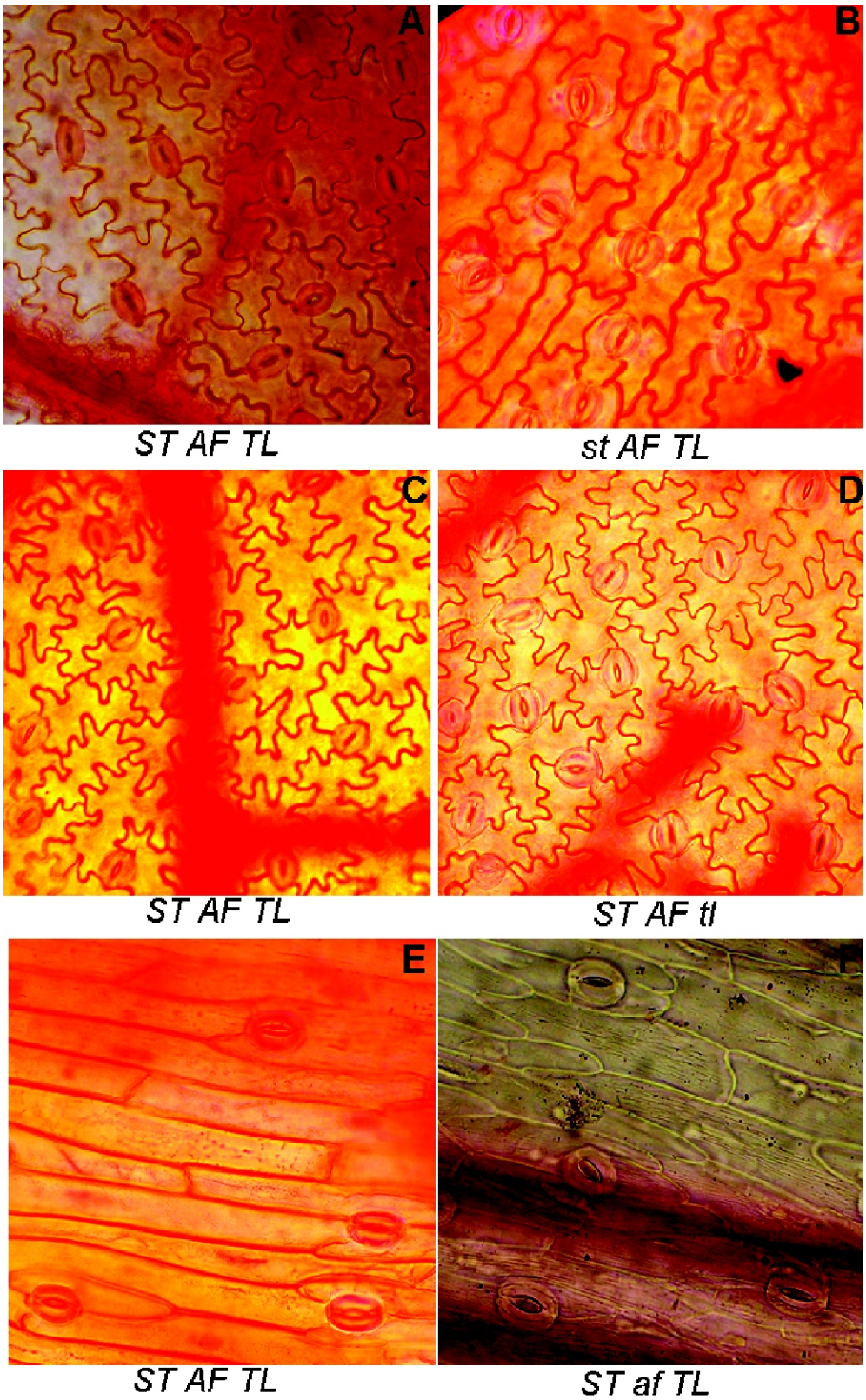

Fig. 5: Epidermal phenotypes of stipules, leaflets and tendrils in pea *Pisum sativum*. Dorsal epidermal layer of stipules in *ST AF TL* (A) and *st AF TL* (B) genotypes and leaflets, located most proximal to petiole, in *ST AF TL* (C) and *ST AF tl* (D) genotypes, and epidermis of primary tendrils in *ST AF TL* (E) and *ST af TL* (F) genotypes, visualized after clearing and safranine staining of the organs. Each of the panel covers 0.25mm^2 area of the organ

Table 5: Epidermal and venation characteristics of the stipules and leaves (leaflets and tendrils) of the *ST AF TL*, *st AF TL*, *ST AF tl* and *ST af TL* genotypes of pea *Pisum sativum*

Genotype	Organ	Stomata (mean±S.E.M.)		Pavement/epidermal cells (mean±S.E.M.)		Venation density † µm/mm² (mean±S.E.M.)			Whether inter-secondary veins formed [Yes(Y)/No(N)]	Highest order of free ending vein(s)	No. of branch points/ mm² (mean±SEM) (A)	No. of free veins/ mm² (mean±SEM) (B)	% of freely ending vienlets to branch point no. (Mean±S.E.M) [(B/A) x 100]
		Frequency (per mm²)	Size (µm²)	Frequency (per mm²)	Size (µm²×10)	Primary vein (×10)	Secondary vein(s) (×10)	Higher order veins (×10)	Total (×10)				
ST AF TL	Stipule	168±24	440±33	492±53	188±37	179±6	976±244	333±43	609	Y	Sixth	15±0.7	8±1.6	54.4
st AF TL	Stipule	171±21	505±50	573±47	160±25	139±27	865±340	166±93	392	N	Third	10±4.2	5±1.3	50.5
ST AF TL	Leaflet	190±28	411±36	445±59	207±50	103±1	1053±234	379±41	587	Y	Fifth	15±3.2	7±1.1	43.8
ST AF tl	Leaflet	181±26	321±34	549±34	172±45	103±1	1071±247	382±39	592	Y	Sixth	18±2.5	12±2.0	63.2
ST AF TL	Tendril	85±10	528±66	259±15	369±54	349±20	241±199	0	373	N	Second	3±1.1	1±0.4	38.7
ST af TL	Tendril	80±6	512±65	179±10	536±64	447±1	485±213	0	495	N	Second	3±1.1	1±0.4	37.0
$F_{5, 30}$ value		14**	2	27**	6*	89**	3	25**	37*		138**	26.0**	64.7**	8.8*
CD‡*		38	141	83	100	35	362	1009	115		0.4	4.1	1.6	12.9
CD**		51	190	112	135	47	488	1360	155		0.6	5.5	2.1	17.4

*, ** = F values from analysis of variance following the completely randomized design: * F significant at 5 % level, ** F significant at 1% level; †= visualized from adaxial surface; ‡ =critical difference values, * at 5% and **, 1% level.

and quinternary, respectively (Table 5). The frequency of veinal branch points and free veins was similar in stipules and leaflets (Table 5). In tendrils, the highest order of venation was secondary (Table 5, Fig. 7M-P). The primary venation was most intensive in tendrils; it was about 75% higher than in stipules and leaflets. Secondary venation occurred in the tendrils but at a low level.

The *st* mutation was observed to have significantly reduced the venation density of the stipules; reduction was by about 25% (Table 5). The highest order of venation in *st* stipules was tertiary. The *st* stipules were significantly richer in primary venation but deficient in higher order venation as compared to *ST* stipules. The highest order of venation in *tl* leaflets was sexternary, although venation density in *TL* and *tl* leaflets was similar (Table 5). Like in *ST* stipules where the highest venation density was also sexternary, the frequency of free veins was high in *tl* leaflets. The *af* tendrils had denser venation as compared to *AF* tendrils (Table 5; Fig. 7M-P). In the stipules and leaflets examined, there was strong positive correlation between venation density and frequency of veinal branch points, frequency of veinal branch points and frequency of free veins and venation density and frequency of free veins (Table 6).

(g) Biomass Accumulation and Partitioning

The biomass accumulated in a whole adult plant was arrived at by summation of separate dry weights of root, stem, stipules, leaves and pods (Table 7). Additional parameters used for plant growth were number of nodes/ phytomers and mean biomass of

Table 6: Pearson's coefficients of correlation (*r*) between anatomical features of stipules and leaflets of pea *Pisum sativum*

	Parameter	Venation density (μm/mm^2)	Number of branch points/mm^2
Number/mm^2 of	branch points free veins	0.73**	
		0.72**	0.69**

*, ** = significant at 5% and 1% probability levels, respectively.

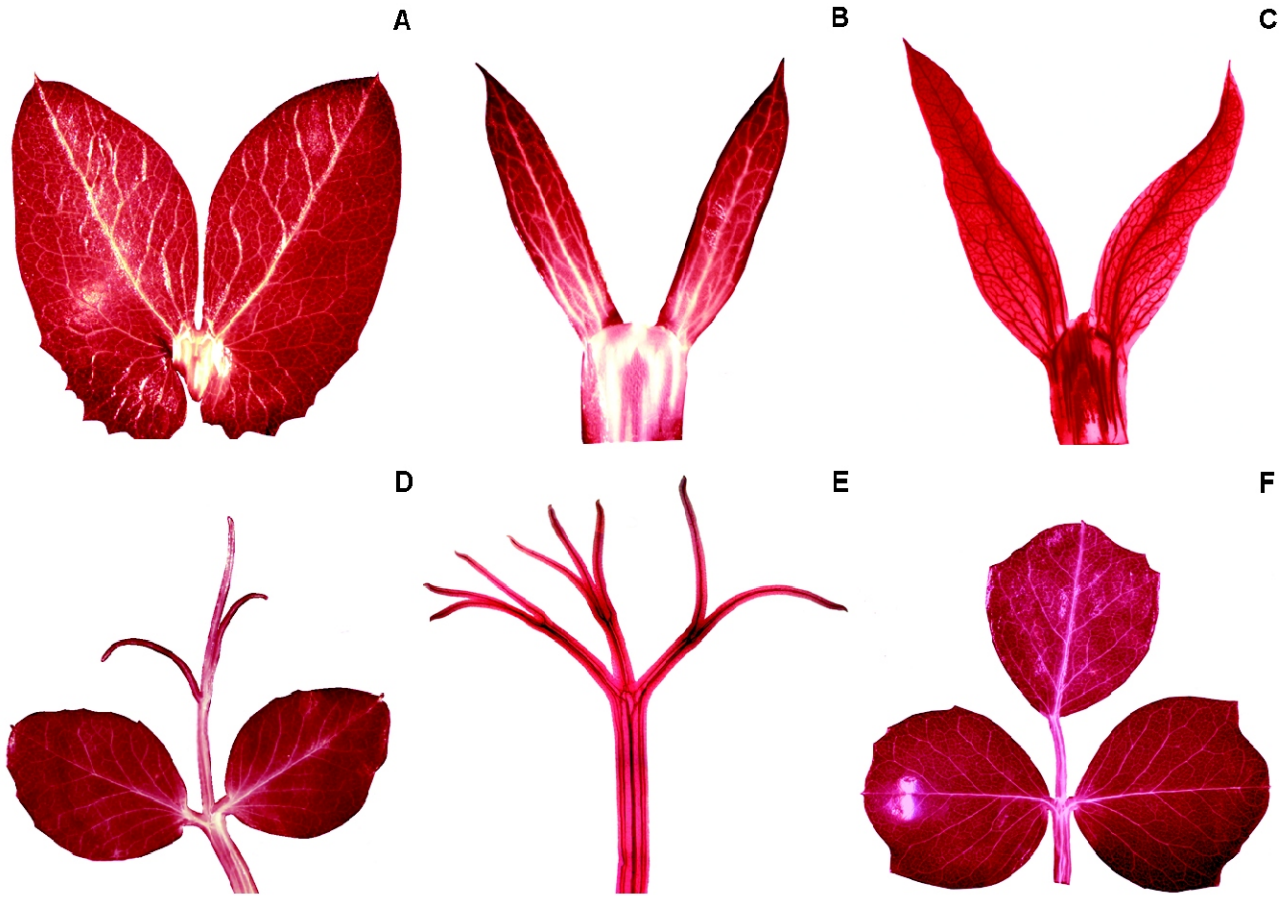

Fig. 6: Patterns of primary and secondary venation visualized dorsally in the cleared whole stipule pairs from *ST AF TL* (A) and *st AF TL* (B and C) and in whole leaves of *AF TL* (D), *af TL* (E) and *AF tl* (F) genotypes of pea *Pisum sativum*. The stipule pairs of *st AF TL* depicted in B and C were taken from juvenile stage node (B) and late flowering stage node (C), respectively. The leaves were taken from the juvenile stage plants. It may be noted that stipules carry one to several primary veins, and 0 to several serrations, depending on the stipule genotype and plant developmental stage

phytomer (Table 7). The variation observed, among the 12 genotypes studied, for all the growth parameters was highly significant. It was noted that the morphologies of stipules and leaves had highly significant impact on the total biomass accumulation. The *coch* and *st* stipule mutations, individually and together, and *af* leafblade mutation had a negative effect on biomass accumulation. On the contrary, the *tl* leafblade mutation had a positive effect on biomass accumulation. Pearson's coefficient of correlation between the root biomass on the one hand and the total biomass and shoot biomass on the other hand were positive and highly significant (Table 8). The correlation of the total biomass with the number of phytomers was similarly high. These results implied that the plant growth was attributable to the

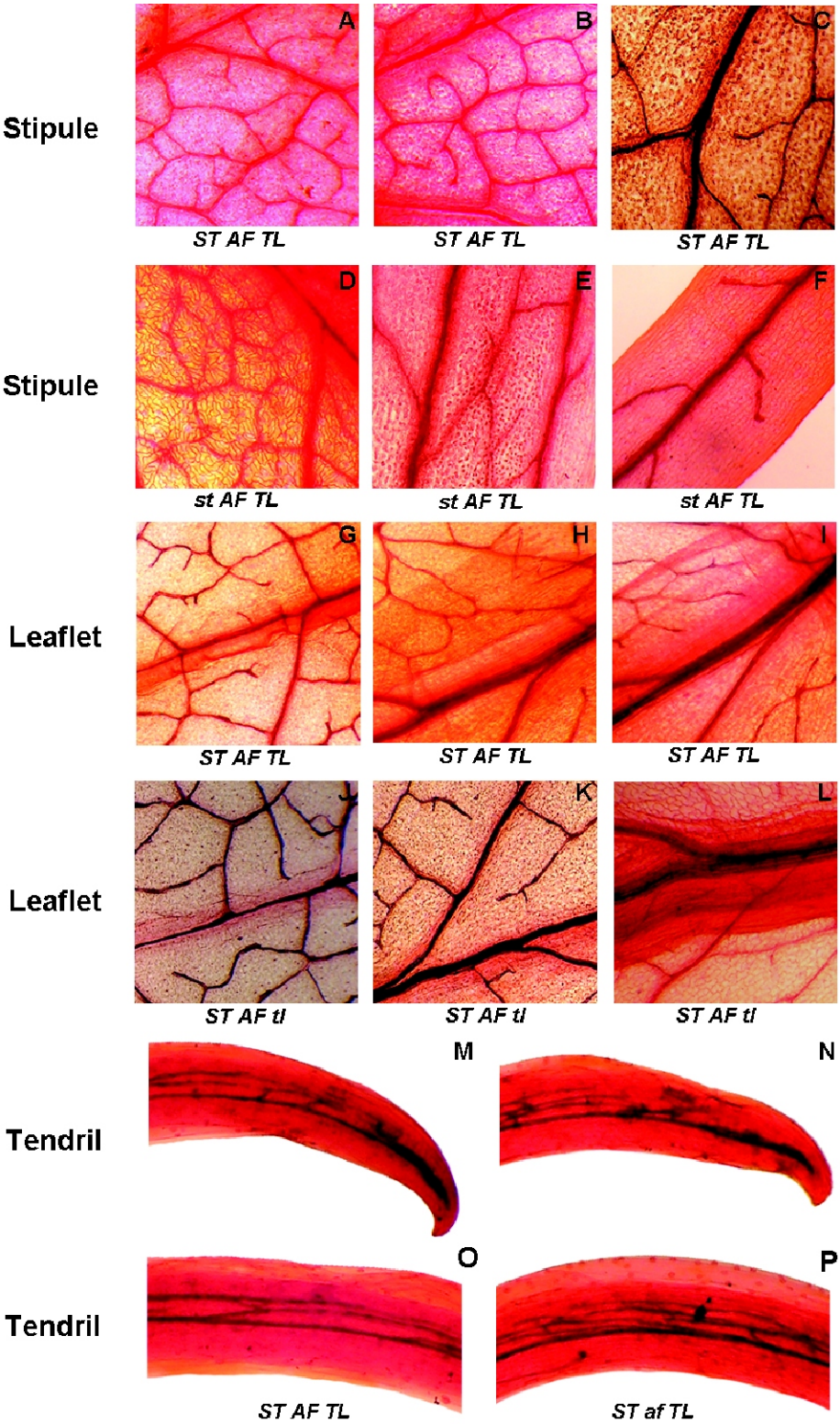

Fig. 7: **Venation phenotype in the stipules, leaflets and tendrils of pea *Pisum sativum*. Shown are the distribution patterns of primary, secondary and higher level veins in the apical (A, G, J, M and N)-, middle (B, D, E, F, H, K, O and P)- and lower (C, I and L)- regions of stipules (A-F), leaflets (G-L) and tendrils (M-P) of the *ST AF TL* (A-C, G-I and M and O), *st AF TL* (D-F), *ST AF tl* (J-L) and *ST af TL* (N and P) genotypes. Venation in the middle regions of stipules, respectively, of type C, D and B of Figure 6 relating to *st AF TL* are shown here in the panels D, E and F. The primary tendrils of *ST AF TL* (M and O) and *ST af TL* (N and P) are depicted. Each of the panel A to L covers 1mm^2 of the lamina area of stipule or leaflet. The tendrils shown in M-P lie in 1mm^2 of the panel area**

Table 7. Effect of variation in the morphology of stipules, caused by *cochleata* (*coch*) and *stipule-reduced* (*st*) mutations and in the morphology of leaves, caused by *afila* (*af*) and *tendril-less* mutations, on the biomass partitioning among organs in *Pisum sativum*

S.No	Genotypes				Total no. of nodes (mean± (S.E.M)(A)	Biomass of (mean ±S.E.M.)									Leaf/stipule ratio (mean± S.E.M.) (H/G)	Leaf and stipule index (mean±S.E.M.) [(G+H/B)x100]	Stem index (mean± S.E.M.) [(F/B)x100]	Havest index (mean± S.E.M.) [(D/B)x100]
	COCH	ST	AF	TL		all the plant organs(g) (B)	a phytometer (g) B/A (C)	seeds (g) (D)	pods (g) (E)	stem (g) (F)	stipules (g) (G)	leaves (g) (H)	only one stipule (mg) (G/A)	only one leaf (mg) (H/A)				
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	+	+	+	+	61±4.1†	29.6±1.31	0.49±0.040	10.67±0.532	14.81±0.896	7.19±0.268	2.28±0.128	5.11±0.400	37.0±1.48	84±7.5	2.2±0.13	25.0±0.75	24.3±1.29	36.1±0.89
2	-	+	+	+	34±4.7	20.6±1.46	0.63±0.044	9.11±1.243	11.92±1.274	3.37±0.242	0.31±0.083	4.82±0.433	9.1±1.21	142±15.2	15.6±2.81	24.9±0.72	16.4±0.98	44.2±3.96
3	+	-	+	+	41±2.5	14.5±0.41	0.36±0.018	4.58±0.390	7.51±0.245	3.49±0.014	0.15±0.026	3.18±0.177	3.6±0.51	78±4.9	21.2±3.40	23.0±0.69	24.1±0.70	31.9±1.79
4	+	+	-	+	38±1.9	8.9±0.33	0.24±0.008	2.72±0.011	3.94±0.041	1.65±0.117	0.89±0.049	2.32±0.172	23.4±1.83	61±3.2	2.6±0.25	36.1±0.86	18.5±0.84	30.6±0.96
5	+	+	+	-	112±10.6	80.7±4.09	0.74±0.046	26.79±1.980	37.52±2.793	18.01±0.582	7.05±0.355	17.47±1.534	63.0±3.19	156±13.4	2.5±0.25	30.4±1.51	22.3±0.90	33.2±1.80
6	-	-	+	+	56±2.6	22.1±0.83	0.40±0.017	2.92±0.589	6.32±0.665	3.51±0.253	0.11±0.028	11.93±0.850	2.0±0.55	230±17.7	108.5±25.6	54.5±3.17	15.9±1.60	13.2±2.44
7	-	+	-	+	48±5.6	14.8±0.91	0.32±0.025	0.55±0.248	1.56±0.474	3.41±0.289	0.20±0.018	9.39±0.955	4.2±0.51	196±11.5	47.0±6.82	64.8±4.28	23.0±2.78	3.7±1.56
8	-	+	+	-	88±2.7	38.3±0.96	0.44±0.015	11.43±0.545	17.29±0.972	9.24±0.837	0.96±0.081	10.38±0.937	10.9±1.20	118±13.2	11.5±1.29	29.6±2.04	24.1±2.26	29.8±2.16
9	+	-	-	+	47±2.6	14.5±1.91	0.31±0.029	4.61±0.436	6.24±0.823	3.04±0.467	0.18±0.036	4.90±0.604	3.8±0.71	104±9.30	27.2±2.91	35.0±1.06	21.0±0.46	31.8±4.11
10	+	-	+	-	69±2.4	29.4±0.89	0.43±0.023	10.11±0.801	13.05±0.184	6.68±0.476	0.44±0.017	8.93±0.528	6.4±0.40	129±11.9	20.3±0.92	31.9±1.16	22.7±1.04	34.4±2.89
11	-	-	-	+	59±2.9	14.5±0.70	0.25±0.007	0.44±0.193	1.85±0.535	3.07±0.421	0.03±0.007	9.29±0.785	0.5±0.24	157±9.07	309.7±93.54	64.3±4.43	21.2±2.65	3.0±1.37
12	-	-	+	-	73±3.1	32.7±2.09	0.45±0.018	6.51±1.861	8.88±1.787	10.91±1.249	0.26±0.080	12.17±0.894	3.6±1.33	167±6.6	46.8±16.52	38.0±1.55	33.4±3.31	19.9±5.30
	$F_{11, 48}$				25**	136.0**	29.08**	56.45**	70.87**	76.12**	280.75**	33.78**	201.1**	21**	9.5**	41.2**	6.4*	22.4**
	CD‡**				13	4.7	0.07	2.72	3.29	1.54	0.34	2.22	3.8	32	125.6	6.8	5.2	7.9
	CD**				17	6.3	0.10	3.62	4.39	2.05	0.45	2.97	5.1	42	167.5	9.0	6.9	10.5
	Range				34-112	8.9-80.7	0.24-0.74	0.44-26.79	1.56-37.52	1.65-18.01	0.03-7.05	2.32-17.47	0.5-63.0	61-230	2.2-309.7	23-67.1	15.9-33.4	3.0-44.2
Mean of all genotypes					60±2.8	27±2.5	0.4±0.02	7.6±0.93	10.9±1.26	6.1±0.61	1.1±0.26	8.3±0.57	14±2.4	135±5.9	51±11.2	38±1.9	22±0.6	26±1.7

Table 7 contd ....

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
COCH genotypes					61±5.3	30±4.8	0.4±0.03	9.9±1.62	13.9±2.25	6.7±1.09	1.8±0.49	7.0±1.03	23±4.3	102±7.2	13±2.1	30±1.0	22±0.4	33±0.4
coch genotypes					60±2.9	24±1.8	0.4±0.02	5.2±0.84	8.0±1.57	5.6±0.64	0.3±0.06	9.7±0.49	5±0.8	168±7.0	90±20.7	46±3.2	22±1.2	19±2.9
$F_{1, 48}$					0.4	36.7**	0.82	74.27**	77.22**	12.53**	495.12**	39.49**	552.5**	110.2**	21.5**	144.9**	0.1	79.9**
ST genotypes					64±6.3	33±4.8	0.5±0.03	10.2±1.69	14.5±2.34	7.2±1.10	2.0±0.48	8.3±0.99	25±4.1	126±8.7	14±3.2	35±2.8	21±0.6	30±2.5
st genotypes					57±2.4	21±1.6	0.4±0.01	4.9±0.63	7.3±0.67	5.1±0.58	0.2±0.03	8.4±0.67	3±0.4	144±9.9	89±20.7	41±2.8	23±1.9	22±2.3
$F_{1, 48}$					5*	130.9**	49.64**	94.13**	115.85**	42.55**	658.15**	0.49	771.7**	6.1*	20.8**	24.9**	2.0	19.6**
COCH ST genotypes					70±10.0	40±9.6	0.5±0.07	13.5±3.16	18.8±4.42	9.0±2.15	3.4±0.83	8.3±4.08	41±5.2	100±13.4	24±0.1	31±1.4	22±0.8	33±0.7
coch st genotypes					63±2.4	23±2.4	0.4±0.03	3.3±0.79	5.7±0.92	5.8±1.14	0.1±0.03	11.1±0.41	2±0.4	185±10.2	155±25.5	52±3.4	24±2.3	12±2.2
$F_{1, 48}$					4*	153.1**	31.60**	167.82**	191.12**	50.62**	1147.48**	24.38**	1315.1**	84.1**	42.3**	144.9**	1.4	89.4**
AF genotypes					67±3.7	34±3.2	0.5±0.02	10.3±1.16	14.7±1.58	7.8±0.79	1.5±0.38	9.3±0.75	17±3.4	138±7.9	29±5.6	32±1.6	23±0.9	30±1.5
af genotypes					48±2.1	13±0.6	0.3±0.01	2.1±0.45	3.4±0.49	2.8±0.17	0.3±0.09	6.5±0.78	8±2.3	130±12.6	97±32.0	50±3.8	21±0.4	17±3.6
$F_{1, 48}$					49**	405.3**	159.40**	195.61**	252.09**	229.66**	238.96**	36.46**	116.4**	2.0	14.4**	140.9**	3.3	55.0**
TL genotypes					48±2.4	17±1.0	0.4±0.02	4.5±0.59	6.8±0.74	3.6±0.25	0.5±0.12	6.4±0.54	10±2.1	132±10.1	67±16.4	41±2.8	21±0.5	24±2.5
tl genotypes					86±4.2	45±5.3	0.5±0.03	13.7±2.01	19.2±2.8	11.2±1.09	2.2±0.73	12.2±0.83	21±6.3	143±5.4	20±4.3	33±0.9	26±1.2	29±1.5
$F_{1, 48}$					189**	759.9**	68.86**	250.40**	306.31**	532.37**	523.52**	143.98**	165.7**	2.7	6.0*	38.2**	21.1**	8.5**
coch af genotypes					54±3.1	15±0.1	0.3±0.01	0.5±0.03	1.7±0.07	3.2±0.08	0.1±0.04	9.3±0.02	2±0.8	177±9.1	178±58.8	65±0.1	22±0.4	3±0.2
coch tl genotypes					81±2.6	36±1.3	0.5±0	9.0±1.10	13.1±1.88	10.1±0.37	0.6±0.16	11.3±0.40	7±1.6	143±10.7	29±0.9	34±1.9	29±2.1	25±2.2
$F_{1, 48}$					37**	159.6**	33.54**	78.62**	96.41**	160.20**	17.61**	6.1*	11.6**	10.2**	25.0**	165.2**	13.1**	60.0**
st af genotypes					53±3.4	15±0	0.3±0.01	2.5±0.93	4.1±0.98	3.1±0.01	0.1±0.03	7.1±0.98	2±0.7	131±12.3	169±63.2	50±6.6	21±0.1	17±6.4
st tl genotypes					71±1.1	31±0.7	0.4±0.01	8.3±0.80	11.0±0.93	8.8±0.95	0.4±0.04	10.6±0.73	5±0.6	148±9.4	34±5.9	35±1.4	28±2.4	27±3.2
$F_{1, 48}$					17**	101.2**	33.97**	36.62**	35.68**	112.94**	4.25*	19.47**	4.0	2.7	21.8**	39.1**	15.4**	10.3**

*, ** = F values from analysis of variance following the completely randomized design: * F significant at 5 % level, ** F significant at 1% level; † = Genotype value for each trait/character is average of 5 replications ± standard error; ‡ = critical difference values, * at 5% and **, 1% level

morphologies of stipules and leaf. The high levels of growth or biomass accumulation were associated with the presence of stipules of large lamina and leafletted leaves as in *COCH/coch ST TL* genotypes or tendrilless leaves as in *tl* genotypes.

The pods, leaves and stipules together, stem and root comprised, on an average basis, of about 0.41, 0.35, 0.23 and 0.01 of the total biomass respectively; and stipules, leaves and seeds comprised of about 0.04, 0.31 and 0.27 of the total biomass, respectively (Table 7). The pattern of biomass partitioning was observed to be significantly affected by the stipule morphology and the leaf morphology. The stipule biomass was decreased by both *coch* and *st* mutations; it was least in the *coch st* genotypes. Reduction in the stipule biomass was accompanied by increase in leaf biomass. Whereas the increase in leaf biomass consequent to *st* mutation was marginal, that from *coch* mutation and *coch st* mutations was highly significant. The mean proportions of stipule biomass: leaf biomass in *COCH ST* and *coch st* genotypes were 41 mg : 100 mg and 2 mg : 185 mg, respectively. The leaf biomass/stipule biomass ratios for *COCH* : *coch*, *ST* : *st* and *COCH ST* : *coch st* were about 13 : 132, 14 : 131 and 3 : 237, respectively. The increase of leaf biomass in stipule morphology mutants was accompanied by the lowering of pod/seed biomass. The seed biomass in *coch*, *st* and *coch st* mutant genotypes was, respectively, about 2.6-, 2.8- and 4.1 fold lower than in *COCH ST* genotypes. Both *af* and *tl* mutations affected the leaf biomass significantly, albeit in opposite directions. The leaf biomass was about 1.5 fold lower in *af* genotypes as compared to *AF* genotypes, and it was about 2 fold higher in *tl*

Table 8: Pearson's coefficients of correlation (*r*) between various parameters of biomass accumulated and partitioned among the major organs in adult pre-harvest plants of twelve genotypes of pea *Pisum sativum*†

Biomass parameter (g)	Root	Stem	Stipule	Leaf	Pod	Seed
Total	0.89**	0.97**	0.90**	0.78**	0.96**	0.94**
Root		0.94**	0.65*	0.89**	0.75**	0.73**
Stem			0.82**	0.77**	0.89**	0.88**
Stipule				0.55	0.91**	0.90**
Leaf					0.58*	0.54
Pod						1.00**

Parameter	Number of nodes	Biomass (g) of		
		phytomer	shoot	root
Total biomass (g)	0.91**	0.81**	1.00**	0.89**
Number of nodes		0.55	0.91**	0.94**
Phytomer biomass (g)			0.81**	0.62*
Shoot biomass (g)				0.89**

Index (percent) parameter	Stipule + leaf	Stem	Seed
Leaf	0.98**	-0.10	-0.94**
Stipule + leaf		-0.13	-0.95**
Stem			-0.10

* = significant at 5 % level; ** = significant at 1 % level; † = Genotypes are listed in Table 7

genotypes in comparison to *TL* genotypes. The seed biomass was about 5 fold higher in *AF* genotypes than in *af* genotypes and 3 fold higher in *tl* genotypes as compared to *TL* genotypes. While the allocation to stem in *ST* versus *st*, *COCH* versus *coch*, *COCH ST* versus *coch st* and *AF* versus *af* genotypes was similar, the *tl* mutation was observed to have increased the biomass allocation to stem by about 25% in *tl* genotypes over *TL* genotypes. The coefficient of correlation between leaf-/stipule+leaf-index and seed harvest index was negative and highly significant, though that between leaf-/stipule+leaf-index and stem index was also negative, but it was marginal (Table 8).

4. Discussion

The work described provides experimental evidence for several functional properties of stipules in *P. sativum*. It is proved that stipules are a major photosynthetic organ type; the stipule anatomy is accordant to that of plant leaves/pea leaflets. Stipules alone, in the absence of adult leaves, can sustain life cycle. Genotypes deficient of stipules are not lethal and produce progeny of smaller size. Regular occurrence of stipules on plant nodes is essential for the realization of plant's biomass production potential. Balanced partitioning of biomass among the vegetative and reproductive organs, for the attainment of high seed harvest index or avoidance of partial sterility, is also dependent on normal stipule development. Aspects of these salient results are discussed below.

(a) Stipules are Effective Photosynthetic Organs

In the wild type *COCH ST AF TL* genotype of pea, the photosynthesis rate in stipules was only about 18% lower than in leaves, and for a phytomere, the stipules were responsible for more than one third of the total photoassimilation (leaf : stipule : stem :: 1 : 0.56 : 0.09). Thus, it emerges that stipules are an important photosynthetic organ. Further, the observation that the plants from which large majority of leaves had been surgically removed formed seed bearing pods, showed that stipules are sufficient to meet the photoassimillate requirements of plant for the completion of its life cycle.

The efficiency of photosynthesis in leaves is known to be not only dependent on the functional activities of the various enzymes of the photosynthetic pathway but also on the structural attributes of its tissue components [34, 35]. In pea leaves, leaflets are the major photosynthetic sub-organs. A comparison of *COCH ST AF TL* stipules and leaflets showed that their laminae were largely similar in adaxial epidermis. The pavement cells of both the organs had similarly irregular boundary. Both the organs were similarly furnished with stomata. Like in the leaflets, palisade- and spongy-mesophyll, parenchyma cells comprised of photosynthetic tissues in stipules. The stipule laminae were marginally thinner than the leaflet laminae because they were short of one layer of spongy parenchyma cells. By possessing the palisade- and spongy- mesophyll cells, the stipules had typical features of leaves of angiospermic dicotyledonous plants. The vascular system of the stipules was highly extensive like that in the leaflets, in terms of intensity of venation reflected by several parameters such as venation density and highest order and frequency of free ending veins. The highest vein order borne in the stipules was sexternary as compared to quinternary in the leaflets. Considering that stipules are short of one spongy parenchyma layer and highest order of veins in them is sexternary, stipules must have a network of interconnected veins much more expounded than in leaflets. Being simple and sessile and directly attached to stem, stipules are apparently provided with an efficient hydraulic architecture, perhaps better than that for compound leaf.

Briefly, the demonstration of occurrence of substantial net photosynthesis in stipules, sufficiency of stipules for the sustenance of life cycle and the presence of stomata in high frequency, typical palisade- and mesophyll- photosynthetic tissues and extensive vascular system prove that stipules are an effective photosynthetic organ, largely similar in photosynthetic characteristics to leaves.

(b) Stipule Has Pinnate-Cum-Palmate Compound Lamina Structure

In this study, a degree of correspondence was noted between *tl* leaflets and *ST* stipules and between *st*

stipules and *AF* and *af* tendrils. The specific stipule area or specific stipule weight were observed to be higher in the *ST AF tl* genotype than in *ST AF TL* genotype. The specific leaf area or specific leaf weight too were higher in *ST AF tl* genotype in comparison to *ST AF TL* genotype. From an earlier study [31], where the stipules were *ST* but leaves differed according to variety in the recombination of wild type and mutant alleles of the leaf morphology genes *UNI* (or *UNIFOLIATA-TENDRILLED ACACIA*, *UNI-TAC*), *AF*, *TL* and *MFP* genes, the estimated coefficient of correlation between stipule size and leaf size was found to be positive and highly significant. Interestingly, the highest order of the free ending minor veins in the *ST AF tl* leaflets and *ST AF TL* stipules was sexternary. The *ST AF TL* stipules were more like *ST AF tl* leaflets than *ST AF TL* leaflets, in the latter the highest order of free ending veins was only quinternary. It has been earlier shown in *Arabidopsis thaliana* leaves that the occurrence of free ending veins is related to the loss of coordination in the proliferation of ground meristem cells for differentiation into the mesophyll cells on the one hand and procambial strands on the other hand [36, 37]. This means that both in the *tl* leaflets and *ST* stipules the homeostatic mechanism(s) favoured the differentiation of ground meristem into mesophyll cells at the expense of further development of the procambial strands. In the proximal domain of pea leafblade, *UNI* (or *UNI-TAC*) and *AF* gene activities are known to promote the development of leaflets. The meristematic activity(ies) in the leaflet primordium are known to be under the homeostatic control of *INS*, *TL* and *MFP* gene activities. The stipule initiation is known to be under the positive control of *COCH* gene. *ST* gene activity is responsible for the proliferation of meristematic activity in the stipule primordium. The gene activities that keep the stipule determinate remain to be revealed. It is possible to speculate that INS, TL and MFP like activities may be involved in the process.

Whereas each *ST AF TL* or *ST AF tl* leaflet contained only one primary vein, the mid-rib, the *ST* and *st* stipules possessed the mid-rib as well as one to several supernumerary primary vein(s) on either side of the central vein. In the *ST* and *st* stipules and *AF* and *af* tendrils, the primary veins were responsible for 20, 35 and 90% of the vein density, respectively. The stipules were observed to be richer in the content of primary veins in their vascular system, which is a property of the tendrils of the pea plant and palmate leaves in general. Taking into consideration the observations that stipules share anatomical features with *tl* leaflets and palmate leaves, it is suggested that perhaps stipules represent a photosynthetic-organ architecture, which combines the properties of both simple pinnate leaf (leaflet) as well as palmate leaf. Thus, in stipules the richness of major veins provides for efficient bulk flow of solutes and mechanical reinforcement of lamina [38] and the highly dense system of higher order veins facilitates more photosynthesis via greater gas exchange capacity [39-41].

The stem proximal part of *COCH ST* stipule lamina is lobed and serrated/toothed. Serrations in simple leaves have been viewed as fused leaflets, where each tooth is considered as single leaflet [42, 43]. Uniquely, in pea plant, the apparently simple stipule simultaneously demonstrates the properties of simple, palmate and compound leafy structure.

It has been shown that vascular redundancy in palmate leaves confers tolerance of hydraulic disruption that may be caused by mechanical or insect damage to the major vein [44]. By having redundant primary veins, the pea stipules are thought to be appropriately equipped for their role as protective organs for the young leaf.

(c) Photosynthesis in Stipules and Leaves and Plant Growth

Photosynthesis forms the basis of plant growth. Two of the measures of growth in a plant are the total number of phytomeres produced and total biomass accumulated. Stipules and leaves are the major photosynthetic organs in the pea plant. Therefore plant growth in pea is expected to depend on the economic investment of photoassimilates produced in stipules and leaves. The *ST AF TL*, *st AF TL*, *ST af TL*, *ST AF tl*, *st af TL* and *st AF tl* genotypes, included in this study of photosynthesis in pea, varied considerably in their phytomeric photosynthetic

capacity jointly constituted by the sizes of stipules and leaf (leaves). It was observed that both the growth parameters, number of phytomeres and biomass, were significantly correlated with the net photosynthesis in phytomere. The relationship of net photosynthesis per phytomere with phytomere number or biomass was linear for five of the six genotypes. In the *ST AF tl* genotype the relative increase in net photosynthesis of phytomere caused logarithmic increase in phytomere number and biomass. It is inferred that the increase in phytomeric photosynthetic capacity or net photosynthesis in stipules and leaf together caused by the *tl* mutation in *ST AF tl* genotype somehow overcomes any kind of negative control which might be exercised by metabolites on the investment of photosynthates in plant growth. The growth promoting pleiotropic properties in *tl* mutant need to be understood in future by their genetic dissection.

(d) Alternate Mechanisms of Increase in Photosynthesis in tl *Leaves and* st *Stipules*

Increase in net photosynthesis in phytomere organs can result from increased rate of photosynthesis or increase in photosynthetic capacity. The rate of photosynthesis in the leaves of *ST AF tl* and *st AF tl* genotypes was about 30-35% lower than that observed in the *ST AF TL* leaves. The increase in leaf size or photosynthetic capacity compensated the effects of decreased photosynthetic rates in *ST AF tl* and *st AF tl* leaves versus *ST AF TL* leaves. The photosynthetic capacity of *st* stipules was upto 7 fold lower than *ST* stipules because of their very small size. However, increase in their photosynthetic rate partially attenuated the effects of small photosynthetic capacity of *st* stipules. The photosynthetic tissues of *st* stipules must have more competent photosynthetic apparatus than in *ST* stipules. The high photosynthetic rate in *st* stipules than in *ST* stipules might be because of the higher density of chloroplasts per unit volume of palisade- and spongy- mesophylls and relative thinness of lamina. There is evidence in rice that the thinness of leafblade contributed to hybrid vigour of F1 hybrids [45]. The photosynthetic rates in the *ST*/*st* heterozygote stipules are not known. It is hypothesized that the fixation of *st* photosynthetic properties in *ST* stipules by genetic engineering together with *tl* leaves could lead to construction of pea lines of high productivity.

(e) Control of Biomass Partitioning by Stipule Morphology Genes

The proportion of biomass among the various organs in the adult plant of *COCH ST AF TL* genotype was root : stipules : leaves : stem : pods (inflorescence) :: 1 : 8 : 17 : 24 : 50. In the pods the proportion of seeds to vegetative part was 1 : 0.4. The stipules + leaves, stem and seeds comprised 0.25, 0.24 and 0.36 of the total biomass, respectively. The pods and their seeds served as the principal sink for the photoassimilates produced in the stipules and leaves. This pattern of biomass partitioning appeared to be in conformity of the genotype having been selected as a seed grain crop cultivar. The stipule- and leaf- morphology mutations changed the above biomass partitioning pattern highly significantly. The seeds partitioned less biomass in *af* genotypes than in *AF* genotypes. In *af* genotypes the biomass, saved from the seeds, got translocated to the leaves. The biomass partitioning pattern of *af* genotypes versus *AF* genotypes was also true for *coch* versus *COCH* and *st* versus *ST* genotypes. In an earlier study of vegetative state of pea plants [46], the *af tl* plants were observed to partition more biomass to leaves than in *AF TL* plants. Thus, the stem and root of *af tl* plants received less biomass than of *AF TL* plants. The present findings proved that stipule morphology genes *COCH* and *ST* control biomass partitioning among organs.

(f) Feedback Inhibition of Biomass Production in the COCH ST Stipule Double Mutants

It is understood that in the flowering stage of pea plant, the photosynthates produced in the extant stipules and leaves will be concurrently invested for the initiation, growth and development of new phytomeres, utilized for the functioning and maintenance of the already formed organs and deposited in the sinks, especially the sink formed by flower forming and seed developing-pod bearing inflorescences. The sum of biomass of all the organs is a good estimate of the total photoassimilation of carbon achieved by the plant, except for the organic

carbon molecules consumed in the respiratory metabolism for sustaining growth and maintenance of cellular (biomass) structure and function.

In the work being reported here, the whole plant biomass was observed to be proportionate to the stipule plus leaf biomass, in all the three *COCH ST* genotypes and *coch ST AF TL* and *COCH st AF TL* genotypes. Contrastingly, the plant biomass was disproportionately smaller in relation to the stipule plus leaf biomass, in all the *coch st* genotypes. As compared to their counterpart, *COCH ST* plants, *coch st* plants partitioned significantly larger biomass to stipules plus leaves and significantly smaller biomass to seeds. Although stem plays an important role in the translocation of organic molecules to the shoot apical meristem where stipules, leaves and inflorescences are initiated, the stem biomass was lower in *coch st AF TL* than in *COCH ST AF TL* and about equal in *coch st af TL* and *COCH ST af TL* genotypes. The stem biomass index was significantly higher in the *coch st AF tl* genotype than in *COCH ST AF tl* genotype. The lower level of seed biomass in *coch st* seeds than in comparison to *COCH ST* seeds was because the flowers of the former genotypes suffered from defective differentiation and

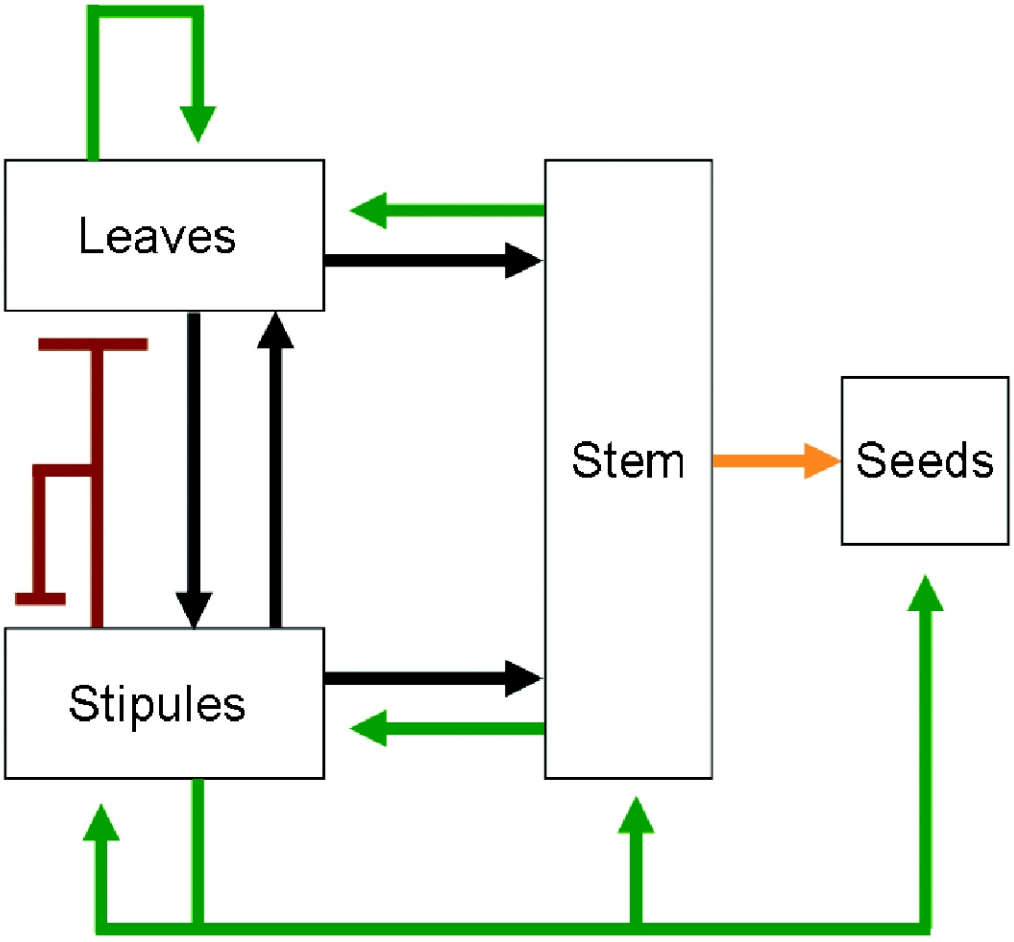

Fig. 8: Diagrammatic representation of the stipular control over biomass -production, -accumulation and -partitioning in pea *Pisum sativum*, suggested by the present study. Both leaves and stipules contribute photoassimilates for the growth of stem (black arrows) and thereby for initiation, growth and development of additional stipules, leaves and seed containing pods (green arrows). Further, presence of stipules promotes flower fertility and therefore normal development of pods/seeds making the latter principal sink for the photoassimilates (dark yellow arrow). Consequently, stipules and leaves remain a minor sink (brown bars), and investment into phytomeric growth continues as per the potential. Genetic deficiency of stipules results in leaves becoming the principal sink and thereby feedback inhibition of photoassimilation ensues together with downregulation of autostimulatory activity in the leaves for photoassimilation. Full growth/photoassimilation/biomass production is not realized.

consequent low seed set in their pods [6, Kumar *et al*. personal communication). Thus leaves were the principal sink for photoassimilates in the *coch st* genotypes. Since the number of phytomeres in the plants of *coch st AF TL* and *coch st af TL* genotypes did not increase over and above the phytomeres in their counterpart *COCH ST* genotypes, there was no extraordinary investment of photoassimilates into the plant growth. The *coch st AF tl* genotype presented an exception in this regard wherein phytomere number was higher than in *coch st AF TL* and *coch st af TL* genotypes but significantly less than in its counterpart *COCH ST AF tl* genotype. Overall, in the absence of seed-sink in its full volume, the potential for biomass production could not be realized maximally. Since the mass of stipules and leaves could not increase further, the non-realization of the full biomass potential must have been due to the attainment of saturation level of biomass in the available stipules and leaves. These observations suggested that *COCH* and *ST* functions are required to prevent feedback inhibition for the production of biomass (organic carbon molecules) in stipules and leaves via photosynthesis. The findings of the study discussed above are diagrammatically represented in Fig. 8.

(g) Essentiality of Stipules in Full Strength for High Harvest Index

Harvest index-wise the genotypes fell into four significantly different groups: *coch st af TL* and *coch ST af TL* (3.3%) < *coch st AF TL* and *coch st AF tl* (16.5%) < *coch ST AF tl*, *COCH ST AF TL*, *COCH ST af TL*, *COCH ST AF tl*, *COCH st AF TL*, *COCH st af TL* and *COCH st AF tl* (32.5%) < *coch ST AF TL* (44.2%). The stipule-deficient *coch st* genotypes demonstrated low harvest index (< 20%), irrespective of the allelic status of leaf genes. Among the genotypes that had high harvest index (≥ 29.8%), only three genotypes gave grain yield significantly higher than the average grain yield of all the 12 genotypes: *COCH ST AF tl* (26.8g/plant), *coch ST AF tl* (11.4g/plant) and *COCH ST AF TL* (10.7g/plant). The stipule occupancy is 100% in *COCH ST* and *coch ST* genotypes [7 and present study], and in comparison to simple and large stipules of *COCH ST* genotypes, *coch ST* genotypes produce simple leaf- or leaflet-like or compound leaf-like stipules. *AF TL* leaves are proximally leafleted with distal tendril and *AF tl* leaves are entirely leafleted and have no tendrils. The observations on harvest index and grain yield allow the conclusion that the presence of *COCH* or *coch* stipules in full strength and leafleted leaves (*AF TL* or *AF tl*) are the requirements for combining high grain yield together with high harvest index.

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