Indian Journal of Agricultural Sciences **84** (1): 93–100, January 2014/Article

Self-incompatibility evidenced through scanning electron microscopy and pollination behaviour in *Stevia rebaudiana*

ASHOK KUMAR YADAV[1], S SINGH[2] and RAJEEV[3]

CSIR-Institute of Himalayan Bioresource Technology, Palampur, Himachal Pradesh 176 061

Received: 17 December 2012; Revised accepted: 4 October 2013

ABSTRACT

Floral development of stevia [*Stevia rebaudiana* (Bertoni)], as followed by light and scanning electron microscopy (SEM), was differentiated into 9 distinct stages, each having distinct landmark features. Tetrad formation takes place at stage-2 and microspore production starts. Highest pollen viability (72.8 per cent) was observed at stage 4, whereas, pollen grain production was recorded to be highest at stage-5. The stages-4 & 5 can be best utilized for pollen collection by dissecting out anthers for manual pollination as the pollen viability was recorded to be highest with maximum pollen production. These stages are important for pollen collection to achieve hybridization and get good seed set. Based on viable seed formation and per cent germination in different pollination treatments studied, pollinations using bumble bee thorax was most successful. No stevia pollen was observed to be dispersed at 2m away from the plant. The results indicate low pollen dispersal through air suggesting entomophilous pollination behavior of the crop. Whereas, no seed set was observed through selfing with bagging as well as selfing by hand-pollination. None of the self pollen adhering to the stigma was found germinated while emerging through anthers in a selfed flower. Although, a good seed set was observed in open pollination suggesting self-incompatibility in the crop.

Key words : Floral biology, Pollination, Pollen dispersal, Scanning electron microscopy (SEM), *Stevia rebaudiana*

Stevia rebaudiana (Bertoni), member of Asteraceae family and only one out of 230 species in the stevia genus that gives the sweetest essence (Soejarto *et al.* 1983). Diterpene glycosides (stevioside and rebaudiosides), produced in the leaves of stevia are high-potency sweeteners about 300 times sweeter than sucrose (Lyakhoukin *et al.* 1993, Matsui *et al.* 1996, Megeji *et al.* 2005, Rajasekaran *et al.* 2007, Robinson 1930, Soejarto *et al.* 1983). These sweet compounds pass through the digestive process without chemically break down, making stevia safe for those who need to control their blood sugar level (Strauss 1995). With the increased incidence of diabetes worldwide and consumer interest in natural products over the synthetic sweeteners, there is a need for improvement of stevia (a natural, non-calorie sweetener) with acceptable taste and health properties (Midmore and Rank 2002). Stevia is a photoperiod sensitive, short day plant, flowers from September to December in northern hemisphere and January to March in the southern hemisphere (Chalapathi 1997). Also, it is reported as highly cross pollinated and entomophilous plant (Miyagawa *et al.* 1986, Oddone 1997).

Plant breeding efforts in stevia have been largely focused on improving leaf yield, prolonged vegetative phase (delayed flowering) and glycosides content particularly rebaudioside-A concentration (which does not have after bitter taste) in the leaves. Concerning the reproductive mechanisms, stevia is a complex and exciting crop because of its numerous particularities. It is a hermaphroditic species but highly cross pollinated, photoperiod sensitive, entomophilous crop (Oddone 1997), which produce self-incompatible (Miyagawa *et al.* 1986) tiny white florets borne in small corymbs of five florets small flowers.

These characteristics affect the success of hybridization and make the improvement of stevia complex. To achieve successful hybridization, a thorough knowledge of flowering and pollination behavior of the plant is required. A detailed morphological description of flower development would therefore provide a vital foundation for future studies that would ultimately give a better understanding of the mechanisms that regulate highly cross pollinated behavior of the crop. In this study, our objective is to identify various developmental stages of flower morphology of stevia and to associate timing of corolla growth and anthesis with stigma

[1]Scientist (e-mail: ashok@ihbt.res.in); [2]Senior Scientist (e-mail: sanatsujat@ihbt.res.in); [3]e-mail: dhiman.22@gmail.com

receptivity and pollen viability which can help in increasing the efficiency of the hybridization and breeding programs.

MATERIALS AND METHODS

Flower development was studied in open-pollinated stevia variety Madhurguna at Stevia Breeding Field at Chandpur Research Farm, CSIR-Institute of Himalayan Bioresource Technology, Palampur (HP). Observations were made on stevia plants from onset of flowering from September to November. The experimental field was divided into three blocks. Fifteen floral buds per plant were tagged on 20 plants in each block at an early developmental stage. The study was conducted at Institute of Himalayan Bioresource Technology (CSIR), Palampur, Himachal Pradesh (India) located at 32°N, 76°E and 1300m above msl. Agro-climatically, the location represents the mid-hill zone of Himachal Pradesh (zone-II) and is characterized by humid sub-temperate climate with high mean annual rainfall (~2500mm). The average air temperature and relative humidity were recorded with a RH Temp-110 data logger, Madge Tech Inc., USA.

Floral buds were observed daily between 9.00 AM and 11.30 AM. We followed the course of events through the period of flower initiation, development, differentiation and divided the developmental process into 9 specific stages and 4 sub-stages. In addition to this, we studied pollen production, pollen viability and stigma development and receptivity with the temporal sequence. Based on the observations in the field study, flower ontogeny of stevia was divided into nine developmental stages: 1) Immature bud stage, 2) Corolla enclosed within calyx, 3) Open ended calyx with visible floret heads, 4) Florets are equal to the height of the calyx but still with closed corolla, 5) One or two florets with increased height and partially open corolla, 6) Fully open florets (further subdivided into four sub-stages), 7) Senescent stage at which corolla tissue collapses, flower loses its shape and white color, 8) Seed developed and pappus bristles increased in size and 9) Mature seed and pappus bristles gets dried. The various floral development stages were identified and photographs were taken under microscope equipped with a camera (Fig 1). Time taken was recorded from one developmental stage to another. Floret length was recorded at various stages of its development so as to determine its full floral cycle.

Stigma and anthers were dissected out from various stages of floret development. Few early stages were dissected under stereo microscope because of very small size of the flower bud. Data was recorded on length of anthers at various developmental stages as well as on length and span of the stigma. Data has been used to observe development of anther and stigma with floret development stages (Fig 2a and b).

Isolation of anthers and stigma from developing inflorescences was performed under a stereomicroscope in small petri dishes containing distilled water (Kamenetsky 1994). Specimens to be used for scanning electron microscopy (SEM) were fixed in 2.5% glutaraldehyde in a 0.2 M phosphate buffer, pH 6.8 (Gabriel 1982, Silva *et al.* 2010), and post-fixed with 1% osmium tetra-oxide (OsO_4) in 0.2M phosphate buffer, pH 7.4 (Brukhin *et al.* 2003) and dehydrated in a graded acetone series (35%, 50%, 70%, 90% and 100%). Immediately thereafter, tissues were dried and specimens were then mounted on aluminum SEM stubs using double-sided carbon tape. The specimens were coated with gold by sputter coating unit at 10 Pascal vacuum for 10 second (E1010 ion sputter Hitachi, Japan) and examined under scanning electron microscope (S-3400 N, Hitachi, Japan). The image was captured on VP-SEM mode at desired magnification at an accelerating voltage of 20 kV.

To determine the pollen production and viability at different floral developmental stages in stevia, florets of different stages were taken in replicate from different flowers and selected plants. Florets were dissected and all the five anthers from each floret were gently removed and vortexed in an eppendorf tube containing 1ml of 0.5M sucrose solution. Sample was vortexed vigorously and the pollen suspension (0.5μl) was taken into a hemocytometer and the number of pollen grains within the counting area was determined. The total number of pollen grains produced per floret was estimated at different floral developmental stages (Fig 3c).

Pollen viability is needed to be assessed so as to pollinate at right time while making crosses. Pollen dehiscence in stevia takes place from early stages of flower development because of which pollen viability test at anthesis do not provide a reliable estimate of pollen viability stage. For this reason, anthers of various stages of florets were crushed on a glass slide, stained and analyzed under microscope. Two staining methods were tested for reliability of results and utilized, one by 1,2,3-triphenyl tetrazolium chloride (TTC) (1% by weight in 50% sucrose) and second by acetocarmine. Data was recorded and analyzed to assess pollen viability stage in stevia (Fig 3d). Pollen viability was also tested by '*in vitro*' germination method.

Pollination experiments included five pollination methods to test for seed set under artificial pollination i) transferring pollens on stigma through forcep, ii) pollination through fine brush, iii) pouring pollens from open flower on stigma, iv) applying mature ruptured anthers on stigma, v) transferring pollen using thorax of bumble bee (*Bombus impatiens*) (entomophily treatment). Likewise, seed set in self-pollination was observed using 1) selfing by bagging (without hand pollination), 2) selfing by hand-pollination with above mentioned pollination methods applying the pollen of the same plant along with control in which plants were left for unmanipulated, natural pollination. For selfing without hand pollination treatments, inflorescences were bagged with nylon mesh before anthesis. Cross-pollination and self-pollination by hand was accomplished by transferring pollen with a bumble bee thorax on the end of a toothpick. The gentle rubbing of stigma with a fine brush/bumble bees for

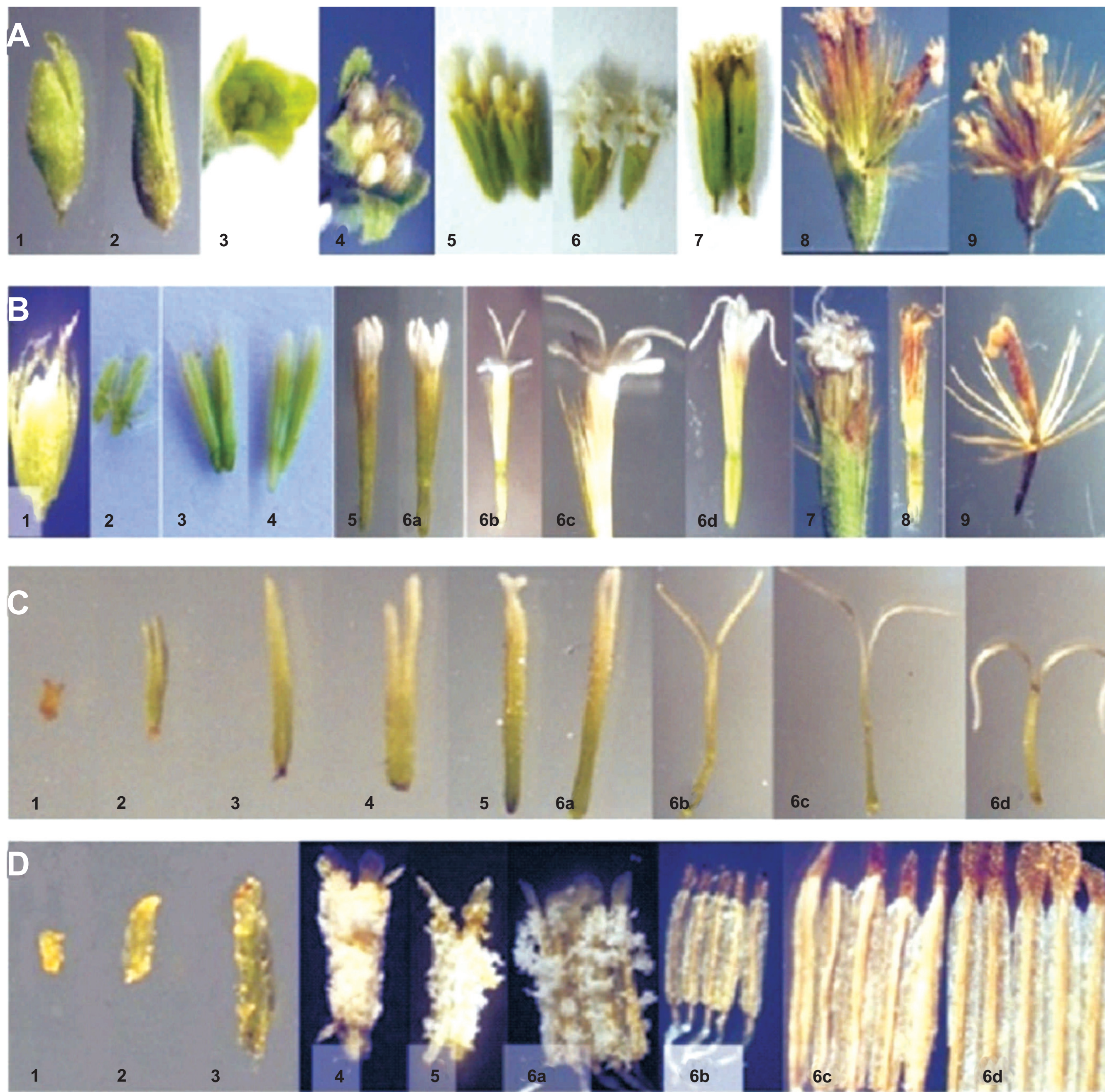

Fig 1 Various floral developmental stages of stevia. A) flower, B) floret, C) stigma and D) anthers

transferring pollens might rupture the cuticle, thus simulating the action of natural pollinators. In the flowering season, individual stevia plants were selected and bagged. All the open flowers were removed before bagging. Data were recorded on three parameters, viz. seed formation, viability and germination (Fig 3a) and were statistically analysed using student's t-test. Pollen dispersal in air was studied near base of the plant, 1m and 2m away from the plant on ground level and at plant height (Mulugeta *et al.* 1994). Pollen in air was counted by placing the microscopic slides smeared with thin layer of glycerine at different locations as mentioned above. Means ± standard error of the mean were calculated for all measurements (Fig 3b).

RESULTS AND DISCUSSION

Floral induction and development

The inflorescence of stevia is loosely paniculate. They are arranged in indeterminate heads. The flowers are small (15-17 mm) and white (Dwivedi 1999, Marsolais *et al.* 1998)

with pale purple throat corollas. The tiny white florets are perfect (hermaphrodite) having both male and female organs, borne in small corymbs of two to six florets (Goettemoeller and Ching 1999). Anthers are small, five in number. Stigma is bi-lobed/ bifurcated from the middle and style is surrounded by anthers (Yadav *et al.* 2011). Stevia floral development stages can be differentiated into 9 distinct stages, each having distinct morphological features which are described stage wise accordingly. Such a morphological description has been carried out for widely used model plants, e.g. *Nicotiana tabacum* (Koitunow *et al.* 1990), *Arabidopsis thaliana* (Schneitz *et al.* 1995, Smyth *et al.* 1990), and *Silene latifolia* (Farbos *et al.* 1997). Floral development keys were identified illustrating developmental stages and their corresponding durations for use as visual reference in manual hybridizations for improvement through breeding.

Flower bud was taken as initial stage-1, when floral bud is almost round and calyx is tightly enclosing 5 immature florets which mature at different times (Fig 1). All the floral parts are green including stigma and anthers. Average size of the floral bud in this stage is 0.5mm, while stigma and anthers are of same size, i.e. 0.2mm (Fig 3a,b). Stage-2 appears after 15 days, where flower bud size is increased to an average of 1.0mm. Florets are still inside the closed flower and average size of stigma and anthers at this stage are 0.4mm and 0.5mm, respectively. Tetrads were observed at stage-2. This is the stage where tetrad formation takes place and pollen production starts (Fig 2). After 10 days, stage-3 appears when the flower bud is open with 5 florets visible inside but the florets are still small and greenish in colour. Average size of the floral bud turns to 3.94 mm, whereas, stigma and anthers are 2.47 mm and 1.48 mm long, respectively. The highest growth in size of floral bud and anthers is found between stage-2 and stage-3 as compared to other stages of flower development. Pollen production in stage-3 starts produced pollen grains up to 8567.57per floral bud (1713.51 pollens/ anther). Pollen grains are shred in the cavity between anthers and stigma as stigma is also surrounded by anthers at this early stage. As the stigma

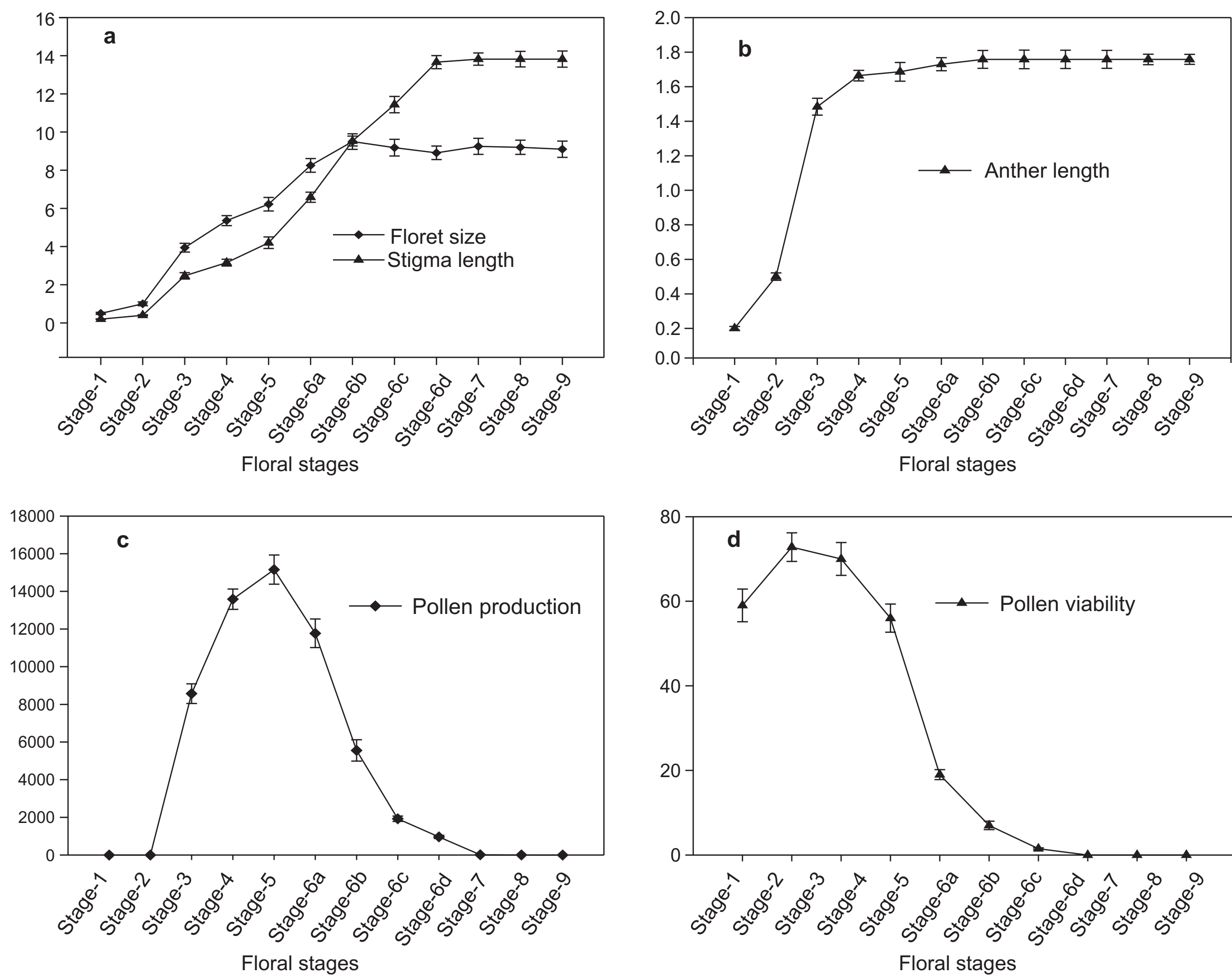

Fig. 2 Variations at different developmental stages in a) floret size (including stigma) and stigma length, b) anther length, c) pollen viability. Vertical bars represent standard error

grows it takes out pollen grains out of this cavity along with its slow movement. Pollen viability test suggested 59.01 per cent viability pointing towards immature stage of pollen grains production (Fig 2d).

Stage-4 appears after 5 days and can be identified distinctly by open flower buds with small white florets visible inside. Average floral bud size increases to 5.36mm, stigma to 3.16mm and anthers to 1.66mm long. Stage-4 was recorded with the highest pollen viability (72.80 per cent) as well as second highest stage for pollen production with 13 585 pollen grains/floret (2 717 pollens/anther) (Fig 2c & d). Stage-5 characterized by one or two white florets increased in length and become ready to open while rest of the florets still remains small and white, which appeared after one day. Average size of larger florets turns out to be 6.22mm. Stigma length at this stage is 4.2mm and anther length remains nearly same as that of last stage, i.e. 1.69mm. Pollen grain production was recorded to be highest in stage-5 with 15 156.00 pollen count/floret (3 031.2 pollens/anther), with 70.00 per cent viable pollens, representing a 2.8 per cent decline in pollen viability. The stage-4 & 5 can be utilized for pollen collection by dissecting out anthers for manual pollination as the pollen viability was recorded to be highest with maximum pollen production which is the major requirement to get good seed set for the breeding programs to be carried out. Also, this pollen can be used in mature pollen culture.

Stage-6 appeared on the next day stage-5 and represents the start of floret opening to fully open flower stage. Since there is a differential growth of florets in a flower, this period can be further divided into four sub-stages depicted as 6a-d according to floret development with minor variation in the floret and stigma only. Sub-stage‘6a’ denotes stage when a floret in a flower starts opening and stigma can be easily seen. This stage appears nearly on the next day of stage-5. Floret length at this stage is 8.25mm (average). Stigma growth is high between stage-5 and this sub-stage, making average stigma length 6.58 mm. Anthers did not show any significant growth in this period and is still 1.73 mm. Average number of pollen grains per anther shows a serious decline and reaches a level of 11 771.33 pollen grains/ floret (2 354.27 pollens/anther) with 14 per cent decline in pollen viability can be seen, showing a total of 56.00 per cent viability at this sub-stage. Pollen viability goes down by 23 per cent and reaches a level of 33.00 per cent if loose pollen collected by simply inverting the floret of same sub-stage is tested. Sub-stage‘6b’ is characterized fully open floret having V-shaped stigma clearly visible outside the floret, making an angle of 60° and average length of floret including stigma is 9.5mm. This stage appears after one day of sub-stage‘6a’. It represents the stage of highest stigma receptivity, which is very important stage for pollination. Anthers did not show any change in their length. Stigma size further increases and reaches a length of 9.53mm. Pollen grain production further declines drastically to 5 719.33 pollens/floret (1 143.87 pollens/ anther) and the pollen viability is reduced to be 19.00 per cent.

Sub-stage‘6c’ can be observed after one day of sub-stage‘6b’. At this stage, V-shaped stigma further expands and makes an angle of 180°. Average length of floret including stigma becomes 9.18mm as the stigma gets flattened. At this stage stigma attains a length of 11.44mm, while no growth in anthers could be seen. Though pollen may be seen in the cavity between style and anthers, but that may be residual pollen, which was not carried out by movement of stigma. Residual pollen grains in this sub-stage are 1 925 per floret (385 pollens/ anther) with 7 per cent pollen viability only. After two days of sub-stage‘6c’, both the lobes of stigma almost loop downwards; this stage is marked as sub-stage ‘6d’. Average length of floret including stigma in this sub-stage is 8.91mm. Sometimes, slight browning can be seen on stigmatic surface, representing areas which are drying. Even at this sub-stage slight growth can be seen in stigma, which reaches a total length of 13.66mm. Residual pollen grains on an average were found 961 pollens/ floret (192.2 per anther) with 1.5 per cent pollen viability. This also marks the end of stage-6.

Stage-7 appears after four days from stage’6d’, when both corolla and stigma starts dry up and turns to pale brown. Ovary turns light black in colour; this marks the stage of initiation of seed development and maturity. Pappus starts growing up and becomes visible. Even at this stage residual pollen can be seen in some cases at the rate of 12.67 pollen grains/floret with no viability. Pappus bristles can be easily seen and starts drying up getting stiffened in stage-8. Corolla gets fully dried up and turn dark brown but calyx can be seen as still light green. Ovary becomes fully dark black colored. Stage-9 marks the drying of whole flower; corolla and stigma both turn dark brown and are ready to detach from ovary. Ovary becomes dark black colored and pappus expands fully. At this stage seed is ready to be dispersed.

Self and cross pollination, seed set and viability

Five pollination methods were tested for seed setting in cross and self-pollination in stevia at an appropriate stage (stage-6b) characterized by V-shaped stigma making an angle of 60° (Fig 1) and average length of floret including stigma 9.5mm after 33 days of bud initiation (Fig 2a). Stage-6b & 6c represents the stage of highest stigma receptivity and can be utilized for pollination. Whereas, stages-4, 5 & 6a are the appropriate stages which produce maximum viable pollen (Fig 2d). These stages should be utilized for collecting the maximum viable pollen for manual pollination in stevia. Among the different pollination methods used for manual cross-pollination data for seed formation, dark colored seed and germination per cent were recorded (Fig 3a). Seed formation (including both pale or clear and dark colored seeds) ranged from 63.33 per cent to 84.43 per cent with highest seed formation though pollination using bumble bee.

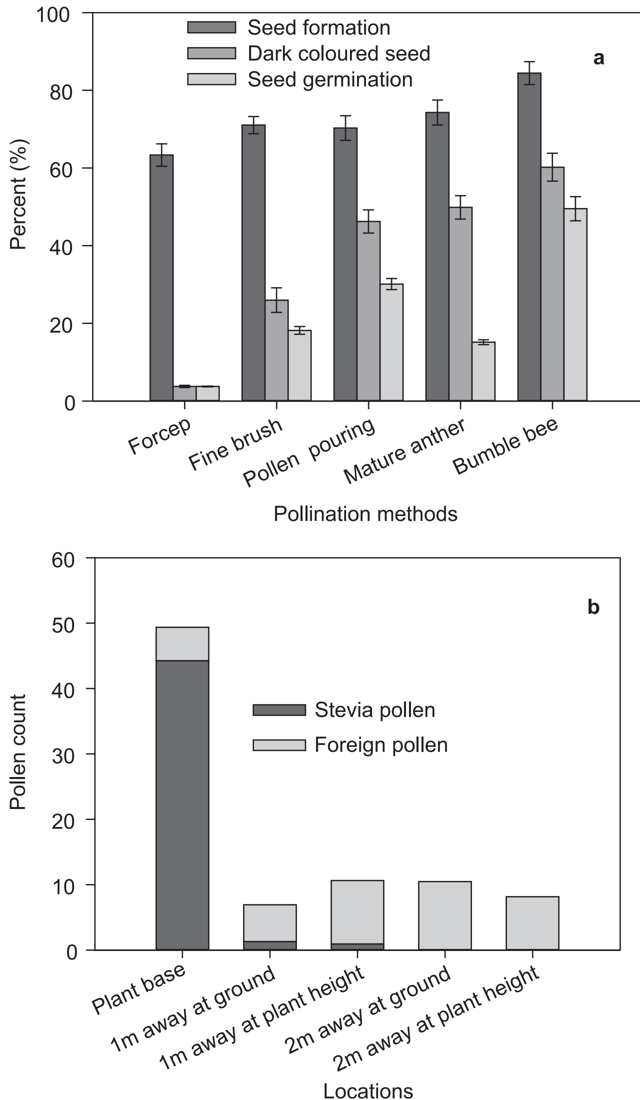

Fig 3 Seed development by different hand pollination techniques (a) and pollen dispersal of stevia (b) Vertical bars represent SE

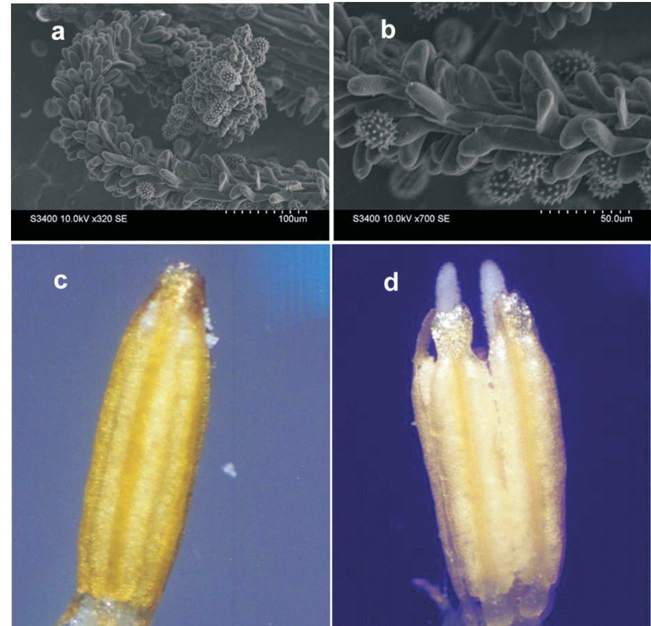

Fig 4 *a-b)* SEM micrographs of *Stevia rebaudiana* showing adhered self-pollen grains on stigma at different magnifications from 30x to 700x and c) micrographs showing attached anthers covering immature stigma, d) stigma emerging out through attached anthers

Among the total seeds developed, dark colored seeds were counted separately. The maximum dark coloured seeds (60.19 per cent) were developed through pollination method using bumble bee followed by applying mature anther to the stigma (49.85 per cent) and the lowest through forcep method (3.75 per cent). Bumble bees have been reported as efficient pollinators in other plant species as well (Nienhuis and Stout 2009). Seed germination ranged from 3.75 per cent with forecep method to 49.51 per cent through bumble bee (*Bombus impatiens*) method. The data for seed characteristics suggested that pollination method using bumble bee was found to be the best method and pollination using forcep performed to be the poorest method (Fig 3a). These results indicated the entomophilous nature of the crop (Miyagawa *et al.* 1986, Oddone 1997).

Likewise, seed set in selfing was observed with bagging and selfing by hand-pollination with above mentioned pollination methods (pollen from same plant) with control. No seed set was obtained in selfing with any of the pollination methods. Although, 48 per cent seed set was observed in control (open-pollinated). The self pollen could be seen adhering to the stigma (Fig 4) as the stigma emerges out through attached anthers. While emerging stigma through the anthers, a huge number of pollens adhere to the stigma surface as anthers mature prior to stigma. None of the self pollen was found germinated on the stigma in a selfed flower suggesting self-incompatibility in the crop. It has been reported that amount of selfing ranged from 0 to 0.5%, while out-crossing ranged from 0.7 to 68.7%, indicating that the self-incompatibility system is operating (Katayama *et al.* 1976, Maiti and Purohit 2008). Similar observations were recorded by others also (Katayama *et al.* 1976, Maiti and Purohit 2008). Similar observations have been made by Miyagawa *et al.* (1986), Katayama *et al.* (1976), Maiti and Purohit (2008). Grashoff (1974) and Monteiro (1980) reported agamospermy in certain genotypes of stevia whereby sexual and apomictic plants of stevia produce normal and malformed pollen, respectively.

Pollen dispersal

Pollen dispersal in air was studied near base of the plant, 1m and 2m away from the stand on ground level and at plant height. Pollen count in air was observed by placing the microscopic slides at different locations as mentioned above.

Near base of the plant average stevia pollen count was highest (44.26) whereas foreign pollen (pollen other than stevia) count was 5.11 pollens (Fig 4b). Average stevia pollen at 1m away from the plant at ground level and at plant height was very low 1.3 and 0.94, respectively. No stevia pollen was observed to be dispersed at 2m away from the plant at ground level or plant height whereas foreign pollens were observed to be more. The results indicate low pollen dispersal through air and support the view that insects are primarily responsible for pollination of stevia as suggested by Oddone (1997) and Miyagawa *et al.* (1986).

CONCLUSION

Nine distinct floral developmental stages and their associated timing were identified with size of corolla growth and anthesis with stigma receptivity and pollen viability which can help in increasing the efficiency of the hybridization and breeding programs. Tetrad formation takes place at stage-2. Stage-5 has the maximum pollen production whereas, maximum viable pollen grains were observed at stage-4. These stages are important for pollen collection to achieve hybridization and get good seed set. Pollination using bumble bee thorax was most successful based on seed development and germination among different pollination treatments. No stevia pollen was observed to be dispersed at 2m away from the plant. The results indicate low pollen dispersal through air suggesting entomophilous pollination behavior of the crop. Whereas, no seed set was observed through selfing with bagging as well as selfing by hand-pollination in the genotypes studied. None of the self pollen adhering to the stigma was found germinated. Although, a good seed set was observed in open pollination suggesting self-incompatibility in the genotype studied.

ACKNOWLEDGEMENT

The authors are grateful to the Director, CSIR-Institute of Himalayan Bioresource Technology, Palampur (H P) India.

REFERENCES

Brukhin V, Hernould M, Gonzalez N, Chevalier C and Mouras A. 2003. Flower development schedule in tomato *Lycopersicon esculentum* cv. sweet cherry. *Sexual Plant Reproduction* **15**: 311–20.

Chalapathi M K. 1997. Natural non-calorie sweetener stevia (*Stevia rebaudiana* Bertoni). Future crop for India. *Crop Research* 14: 347–50.

Dwivedi R S. 1999. Unnurtured and untapped super sweet nonsacchariferous plant species in India. *Current Science* **76**: 1454–61.

Farbos I, Oliveira M, Negrutiu I and Mouras A. 1997. Sex organ determination and differentiation in the dioecious plant *Melandrium album* (*Silene latifolia*): a cytological and histological analysis. *Sexual Plant Reproduction* **10**: 155–67.

Gabriel B L. 1982. *Biological Electron Microscopy.* Van Nostrand Reinhold, Princeton.

Goettemoeller J and Ching A. 1999. Seed germination in *Stevia Rebaudiana*. *Perspectives on New Crops and New Uses,* pp 510–1. Janick J (Ed). ASHS Press, Alexandria, VA.

Grashoff J L. 1974. Novelties in stevia (Compositae: Eupatorieae). *Brittonia* **26**: 347–84.

Kamenetsky R. 1994. Life cycle, flower initiation and propagation of the desert geophyte *Allium rothii*. *International Journal of Plant Sciences* **155**: 597–605.

Katayama O, Sumida T, Hayashi H and Mitsuhashi H. 1976. The practical application of Stevia and research and development data. ISU, Company, Japan, pp 747.

Koitunow A M, Truettner J, Cox K H, Wallroth M and Goidberg R B. 1990. Different temporal and spatiai gene expression patterns occur during anther development. *Plant Cell* **2**: 1 201–24.

Lyakhoukin A G, Long D A and Titoy M P A. 1993. Cultivation and Utilization of stevia (*Stevia rebaudiana* Bertoni). Agricultural Publishing House, Hanoi, pp. 1–44.

Maiti R K and Purohit S S. 2008. Stevia: A miracle plant for human health. Agrobios (India), Jodhpur.

Marsolais A A, Brandle J and Sys E A. 1998. Stevia plant named RSIT 94–751. US Patent, United States.

Matsui K, Kawasaki Y, Oda Y, Noguchi T, Kitagaewa Y and Sawada M. 1996. Evaluation of the genotoxicity of stevioside and steviol using six *in vitro* and one *in vivo* mutagenicity assays. *Mutagenesis* **11**: 573–9.

Megeji N W, Kumar J K, Virendra S, Kaul V K and Ahuja P S. 2005. Introducing *Stevia rebaudiana*, a natural zero-calorie sweetener. *Current Science* **88**: 801–4.

Midmore D J and Rank A H. 2002. A new rural industry - Stevia - to replace imported chemical sweeteners. Rural Industries Research and Development Corporation, Barton, ACT, Australia.

Miyagawa H, Fujikowa N, Kohda H, Yamasaki K, Taniguchi K and Tanaka R. 1986. Studies on the tissue culture of *Stevia rebaudiana* and its components: (II). Induction of shoot primordia. *Planta Medica* **4**: 321–4.

Monteiro R. 1980. Taxonomia e biologia da reproducao da *Stevia rebaudiana* Bert. University of Estadual de Campinas, Brazil.

Mulugeta D, Maxwell B D, Fay P K and Dyer W E. 1994. Kochia (*Kochia scoparia*) pollen dispersion, viability and germination. *Weed Science* **42**: 548–52.

Nienhuis C M and Stout J C. 2009. Effectiveness of native bumble bees as pollinators of the alien invasive plant *Impatiens glandulifera* (*Balsaminaceae*) in Ireland. *Journal of Pollination Ecology* **1**: 1920–7603.

Oddone B. 1997. How to grow stevia. Technical manual. Guarani Botanicals, Pawtucket, CT.

Rajasekaran T, Giridhar P and Ravishankar G A. 2007. Production of steviosides in *ex vitro* and *in vitro* grown *Stevia rebaudiana* Bertoni. *Journal of the Science of Food and Agriculture* **87**: 420–4.

Robinson R L. 1930. Contributions from the Gray Herbarium of Harvard University XC. The Gray Herbarium of Harvard University, Cambridge, pp 78–91.

Schneitz K, Hülskamp M and Pruitt R E. 1995. Wild-type ovule development in *Arabidopsis thaliana*: a light microscope study of cleared whole-mount tissue. *Plant Journal* **7**: 731–49.

Silva E D, Vanzela A L L and Mariath J E A 2010. Developmental and cytogenetic analyses of pollen sterility in *Valeriana scandens* L. *Sexual Plant Reproduction* **23**: 105–13.

Smyth D R, Bowman J L and Meyerowitz E M. 1990. Early flower development in *Arabidopsis*. *Plant Cell* **2**: 755–67.

Soejarto D D, Compadre C M, Medon P J, Kamath S K and Kinghorn A D. 1983. Potential sweetening agents of plant origin. II. Field search for sweet-tasting *Stevia species*. *Economic Botany* **37**: 71–9.

Strauss S. 1995 The perfect sweetener. *Technology Review* **98**: 18–20.

Yadav A K, Singh S, Dhyani D and Ahuja P S. 2011. A review on the improvement of Stevia (*Stevia rebaudiana* (Bertoni)). *Canadian Journal of Plant Science* **91**: 1–27.