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Strategies to improve poor seed germination in *Stevia rebaudiana*, a low calorie sweetener

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Expanding cultivation of exotic 'stevia' in mid- and low-hill areas of Himachal Pradesh, India, is difficult because of the problem of low seed fertility. The objectives of the present study were to devise strategies to produce fertile seed. Two distinct compatible morphotypes were isolated, which when used in pollination produced fertile seeds, whereas self-pollination always resulted in sterile seed set, indicating sporophytic self-incompatibility in the species. To obtain fertile seeds, a mixed population of compatible genotypes should be raised. Although sporophytic chromosome numbers ranging from 22 to 70 are reported in literature, both the morphotypes investigated had only 22 chromosomes but differed on the basis of leaf shape, shoot collar diameter, plant type (compact or loose) and stevioside/rebaudioside A content.

Key words: *Stevia rebaudiana*, chromosome number, pollination, stevioside, rebaudioside A.

INTRODUCTION

Stevia rebaudiana Bertoni (family Asteraceae), a perennial herb commonly known as 'Stevia', is an important source of natural calorie-free sweeteners of which stevioside and rebaudioside A are the most important (Goettemoeller and Ching, 1999; Midmore and Rank, 2002; Singh and Rao, 2005; Dacome et al., 2005; Sekaran et al., 2007; Hadia, Badawy and Hafez, 2008). These sweeteners are 100 to 400 times sweeter than sucrose, the common sugar (Ishima and Katayama, 1976; Kaneda et al., 1977; Ishima et al., 1978; Tanaka, 1982; Sekaran et al., 2007; Yadav et al., 2011; Pande and Gupta, 2013. These natural sweeteners have no adverse effects on human health; on the other hand, being safe for diabetics (Mogra and Dashora, 2009; Misra, 2011), calorie-free and non-toxic, these have beneficial medicinal effects (Elkins, 1997; Srimaroeng et al., 2005), antimicrobial (Satishkumar et al., 2008), antifungal (Silva et al., 2008), hepatoprotective (Mohan and Robert, 2009) and antiviral properties (Kedik et al., 2009). Under *in vitro* conditions, hot water extract of its leaves has been reported to inhibit the replication of all four serotypes of human rotavirus (Takahashi et al., 2001).

S. rebaudiana, native to semi-humid, subtropical region of Paraguay (Shock, 1982a; Oddone, 1999; Jeppesen et al., 2002, 2003; Srimaroeng et al., 2005) has a major problem of very low seed fertility. Study of the literature reveals that there is no agreement on the reasons for the low seed fertility. Some investigators (e.g., Miyagawa et al., 1986; Chalapathi et al., 1997; Oddone, 1997; Maiti and Purohit, 2008) have reported the species to be self-incompatible, whereas others (Goettemoeller and Ching, 1999) did not encounter any self-incompatibility.

The number of chromosomes also is a controversial subject in this species. For example, Monteiro (1980a, 1982), Frederico et al. (1996) and Oliveira et al. (2004)

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have reported 2n=22 chromosomes for this species. However, cytotypes with 2n=24, 33, 34, 44, 48, 66, 70 have been reported by others (Darlington and Wylie, 1955; Bolkhoviskikh et al., 1969; Moore, 1973, 1974, 1977; Goldblatt, 1981, 1984, 1985, 1988; Goldblatt and Johnson, 1990, 1991, 1996, 1998). Monteiro (1980b) reported about 65% pollen viability in contrast to zero percent reported by Oliveira et al. (2004) in a cytotype with 2n=22 chromosomes.

Variation in stevioside content from 3.8 to 18.5% (Sakamoto et al., 1975) and in rebaudioside A from 0.3 (Hashimoto et al., 1978) to 12.1% (Huang et al., 1995) has been reported from different countries. Also, as rebaudioside-A is sweeter than stevioside (Kaneda et al., 1977; Tanaka, 1982), strains with high rebaudioside-A content are preferred to those with high stevioside content (Dacome et al., 2005; Sekaran et al., 2007; Yadav et al., 2011).

The species has been introduced in several mid- and low-hill areas of Himachal Pradesh, India for commercial cultivation. However, poor seed fertility restricts its large-scale commercialization. To address the issue of low seed fertility, this study was undertaken.

Simultaneously, the ploidy status and variation with regard to stevioside and rebaudioside A content in the introduced strains was also studied, because ploidy level may have a bearing on its successful domestication and commercialization in Himachal Pradesh.

MATERIALS AND METHODS

Plants for the study were obtained from a vegetatively propagated population growing at the research farm (1250 m above mean sea level) of University of Horticulture and Forestry, Solan (HP), India. For meiotic analyses, floral buds were fixed in ethyl alcohol:acetic acid:chloroform (1:1:1, v/v/v) for 24 h, washed thoroughly and stored in 70% alcohol. Anthers were squashed using 1% acetocarmine stain. For mitotic analyses, healthy root tips were pretreated with paradichlorobenzene for 4 h and fixed in ethyl alcohol:acetic acid (3:1, v/v) for 24 h, washed and stored in 70% absolute alcohol. Before squashing, the pretreated root tips were hydrolyzed in 1 N HCl at 60°C for 10 min and were transferred to basic fuchsin stain (Maria et al., 2008). Corresponding anther dehiscence and stigma-receptive stages were determined by the usual microscopic and *in situ* pollen-germination methods (Maria et al., 2008). Flowering tops were bagged to achieve self-pollination. In addition, controlled cross-pollination between different plants was accomplished by emasculating the female parent and then dusting the stigma with pollen from other plants. Seed germination was evaluated by germinating seeds in Petri plates at 24°C under alternating light and dark conditions in a growth chamber maintained at about 70% relative humidity.

For chemical analyses, leaf samples from at least ten plants each of the two morphotypes, collected separately, were shade dried followed by oven drying at 60°C till constant weight. 0.5 g of dry leaf powder was first subjected to repeated extraction with chloroform and then with cold methanol (each operation repeated four times) (Pól et al., 2007). The methanol extracts were analysed for stevioside and rebaudioside A content through Water's high performance liquid chromatography (HPLC) system using NH_2 column (4.6 × 250 mm) and acetonitrile:water (80:20) as the mobile phase for elution. Chromadex grade reference compounds of stevioside and rebaudioside A were used.

Data on morphological and chemical parameters studied was calculated as mean+standard error (SE) whereas Randomized Block Design (RBD) statistical tool was used in analyzing pollination experiment data.

RESULTS AND DISCUSSION

In the study population, two types of plants (herein referred to as morphotype A and B), (Figure 1), were observed on the basis of leaf shape, collar diameter of shoot, plant type, etc (Table 1). Though general morphological features of both morphotypes were as per earlier reports (King and Robinson, 1987; Katayama et al., 1976; Shock, 1982a, b; Liu and Li, 1995; Megeji et al., 2005; Schmeling, 1967; Sakaguchi and Kan, 1982; Singh and Rao, 2005), a constant number of five florets per corymb was observed which, however, is different from the varying number of 2 to 6 florets per corymb reported by Goettemoeller and Ching (1999). Although the number of florets per corymb does not have any bearing on seed fertility, the present observation does indicate the presence of different morphotypes in the species based on number of florets per corymb. The flowers of both the morphotypes were found to be protrandrous as anther dehiscence started in unopened florets, whereas stigma receptivity coincided with floret opening and spreading of stigmatic lobes outside the corolla tube (Figure 2 and Table 2). At peak flowering stage, 4.25±0.19% stevioside and 2.01±0.012% rebaudioside A in morphotype A and 7.32±0.029% stevioside and 4.13±0.002% rebaudioside A in morphotype B was estimated. Both morphotypes had n=11 and 2n=22 chromosomes (Figures 3,4 and 5), which is in conformity with reports of Monteiro (1980a, 1982); Frederico et al. (1996); Oliveira et al. (2004) but differs from 2n=24, 33, 34, 44, 48, 66, 70 chromosomes reported by others (Darlington and Wylie, 1955; Bolkhoviskikh et al., 1969; Moore, 1973, 1974, 1977; Goldblatt, 1981, 1984, 1985, 1988; Goldblatt and Johnson, 1990, 1991, 1996, 1998). This indicates the occurrence of different cytotypes in the species and the cytotypes investigated/introduced here are with 2n=22 chromosomes. Chromosomal behavior during meiosis in both the morphotypes was mostly normal, as no abnormalities, such as laggards and bridges, were detected at anaphase-I. However, in some of the pollen mother, cells at diplotene and diakinesis, a few univalents were also observed indicating precocious separation or suspected weak desynapsis which may be the reason for only about 70% pollen viability observed in both the morphotypes. Although Monteiro (1980b) also reported almost similar pollen fertility (65%), Oliveira et al. (2004) observed zero percent pollen fertility in cytotypes with 2n=22 chromosomes. This suggests the prevalence of different ecotypes with varying levels of pollen fertility within the same cytotypes.

Both the morphotypes set two types of achenes (seeds), namely, black and tan colored (Figure 8); the

Table 1. Morphological differences between the two morphotypes of *Stevia rebaudiana*.

Morphotype A	Morphotype B
Compact habit (Figure 1)	Loose habit (Figure 1)
Emergence of multiple shoots more uniform	Emergence of multiple shoots less uniform
Individual shoots less branched	Individual shoots highly branched
Collar diameter of individual shoot was 5.1±0.221 mm	Collar diameter of individual shoot was 13.89±0.563 mm
Leaf shape obovate to ovate	Leaf shape obovate
Leaf tip obtuse (Figure 2)	Leaf tip acute (Figure 2)

Table 2. Corresponding sequence of events leading to anther and stigma maturation.

No. of days since floral bud initiation	Floral stage	Anther	Stigma
19-20	Unopened	Anthers mature and start to dehisce	Stigmatic lobes adpressed and above the anther surface
20-22	Flower begins to open	Maximum anther dehiscence (Pollen shedding)	Style further elongated and stigma well above the anther surface. Stigmatic lobes begun to spread out
22-24	Full blooming stage	Anther dehiscence almost completed but some pollen still adhering to the anther surface	Stigma lobes extrude outside the corolla tube, stigma lobes bifurcated (stigma receptive for pollen germination)
25-27	Flowers begin to dry	Anthers started withering	Stigma lobes fully opened
29-32	Complete drying of flowers	Anthers fully dried	Stigmatic lobes start curling backwards changing color due to drying and lose receptivity

former were heavier, longer and wider than the latter (Table 3). However, development of these two types of achenes appeared to be controlled by a compatibility factor, as black achenes were produced only when plants of both morphotypes (A and B) were involved in pollen transfer, whereas tan-colored achenes resulted from self-pollination in both the morphotypes. As presented in Table 3, controlled crossing between morphotypes (B × A and its reciprocal A × B) gave the highest black seed set of 86.71% (for B × A) and 85.43% (for A × B) with the rest being tan colored seed. Germination test revealed tan-colored achenes to be completely sterile, whereas black achenes showed germination of up to 85% (Figures 6 and Table 3). Low seed fertility under open-pollination, as compared to the controlled pollination, can be attributed to assured viable pollen load at the stigma receptive stage in the latter which was not the case with the former (Table3).

When plants of the same morphotype (A or B) were involved in pollen transfer, no pollen germination took place even when applied at stigma receptive stage. However, when such transfer involved plants of both morphotypes (A and B), abundant pollen are germinated (Figure 7). This indicated that the two identified morphotypes (A and B) were different, but with compatible genotypes. This suggested the prevalence of self-incompatibility barrier in these morphotypes. As the incompatibility barrier was expressed at the stigma-pollen interface, the type of self-incompatibility was regarded as sporophytic, which is in agreement with earlier studies (Miyagawa et al*.,* 1986; Chalapathi et al*.,* 1997; Oddone, 1997; Proctor et al., 1996; Maiti and Purohit, 2008). Goettemoeller and Ching (1999) did not encounter any self-incompatibility barrier in this species which indicated that different clones of this species can either be self-incompatible or compatible. Present studies suggested that a reason of low seed fertility in populations of *S. rebaudiana* grown here was homogenous genetic make-up of the populations. Populations consisting of diverse but compatible genotypes are expected to yield substantial fertile seeds (black-colored achenes). More than 50% black seed set (under open-pollination conditions) was obtained when mixed populations of the two identified morphotypes were raised. Success or failure of pollination, followed by fertilization, was manifested by the degree of fertile seed production, as determined by the germination tests. In *S. rebaudiana*, apart from the germination tests, fertile and infertile seeds can also be determined on the basis of seed color; black-colored seeds being fertile and tan-colored seeds infertile.

The salient conclusions that emerge are that fertile seed production under monoculture conditions in this species would not be possible, which would hamper

Table 3. Effect of different pollination methods on seed set and germination in *Stevia rebaudiana*.

Treatment		Seed set (%)		Seed germination (%)		MDG (%)	GEI (%)	100 Seed weight (mg)	
		Black☼	Tan☼	Black☼	Tan	Black☼	Black☼	Black☼	Tan☼
1	**Self pollination by Bagging**								
T1	Morphotype A	0.00 (0.00)	100.00 (90.00)	-*	0.00	-*	-*	-*	17.53
T2	Morphotype B	0.00 (0.00)	100.00 (90.00)	-*	0.00	-*	-*	-*	18.45
	By Hand								
T3	Morphotype A × A	0.00 (0.00)	100.00 (90.00)	-*	0.00	-*	-*	-*	17.66
T4	Morphotype B × B	0.00 (0.00)	100.00 (90.00)	-*	0.00	-*	-*	-*	18.93
2	**Controlled cross pollination**								
T5	Morphotype A × B	85.43 (67.97)	14.57 (22.03)	82.14 (65.82)	0.00	10.42 (3.22)	58.90 (50.16)	38.13	17.83
T6	Morphotype B × A	86.71 (68.84)	13.29 (21.16)	85.71 (68.56)	0.00	12.08 (3.47)	63.10 (52.64)	39.56	18.97
3	**Open pollination**								
T7	Morphotype B#	0.00 (0.00)	100.00 (90.00)	-*	0.00	-*	-*	-*	18.07
T8	Morphotype A##	60.00 (50.88)	40.00 (39.12)	58.57 (50.12)	0.00	8.95 (2.98)	44.09 (41.58)	35.70	17.50
T9	Morphotype B###	12.28 (20.17)	87.72 (69.48)	60.71 (51.31)	0.00	8.77 (2.94)	45.05 (42.13)	37.54	18.89
$CD_{0.05}$		4.45 (3.29)	4.45 (3.29)	11.49 (7.59)	-	1.87 (0.29)	8.38 (4.85)	1.89	0.89

☼Significant at 5% level of significance; *No observation was taken because of zero black seed set.; #Distanced from morphotype A plants;##Grown close to morphotype B plants; ###Grown close to morphotype A plants. Low black seed set may be due to a very limited stock of morphotype A plants growing in a large field containing mostly morphotype B plants. Number of plants of morphotype A was only 5 that too were scattered unevenly and by chance occurred on the margin of the field. It appears that low black seed set in morphotype B plants may have been due to very limited morphotype A pollen availability; MDG= Mean Daily Germination; GEI = Germination Energy Index. Values given in parenthesis are arc sign transformed values (square root in MDG %).

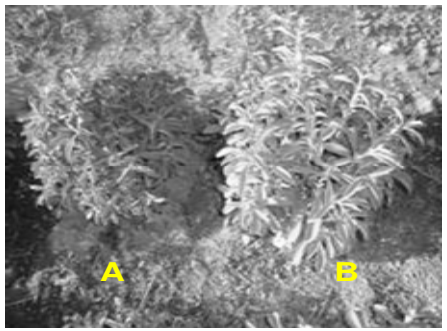

Figure 1. Morphotypes

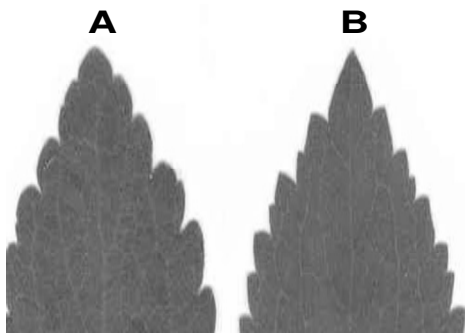

Figure 2. Leaf tip.

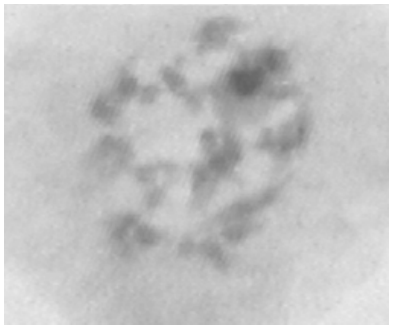

Figure 3. Diplotene

Figure 4. Diakinesis (n=11).

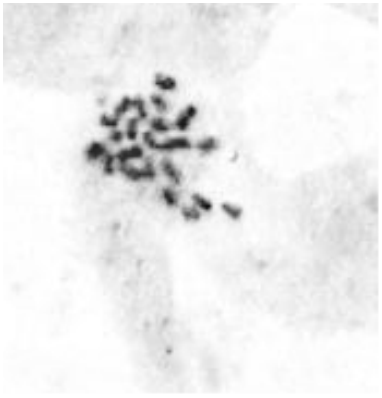

Figure 5. Metaphase (2n=22).

Figure 6. Seedlings.

large-scale fertile seed production and require use of costly vegetative or micropropagation (tissue culture propagation) methods. It is recommended that for production of fertile seeds, a mix of at least two compatible genotypes should be used.

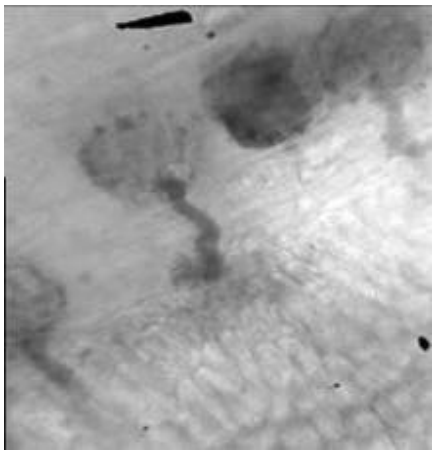

Figure 7. Pollen germination on stigma.

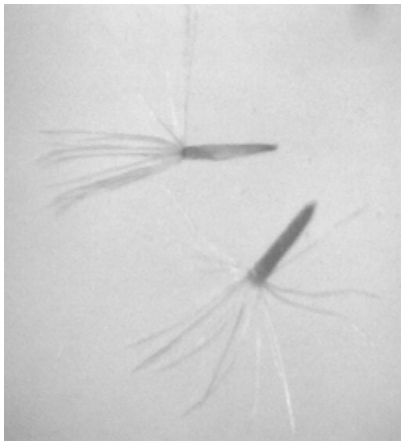

Figure 8. Black and Tan seeds.

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