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Studies on Pollen Viability and Germinability in Accessions of *Stevia rebaudiana* Bertoni

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Abstract

Stevia rebaudiana produces sweet steviol glycosides extractable from the leaves. With zero calorie contents, it represented inevitable health diet for diabetic patients. Poor seed germination (10%) posed obstacles towards large scale establishment. Pollens play fundamental role in fertilization and seed sets. Studying pollen *in vitro* germination and growth of pollen tube are essential for explaining lack of fertility in spermatophytes. Limited studies exist on the pollen profile of this crop. Pollen viability and germinability in *Stevia* accessions MS007, MS012 and SBK were studied. 3000 pollen grains per accession were examined for viability. Evaluated parameter includes pollen staining ability in Cotton blue in lacto phenol. Boric acid concentrations (0.025 g, 0.05 g and 0.1 g) prepared with 20 g of sucrose in 100 ml distilled water were formulated into pollen germination medium (GM), 300 pollens per accession were scored for germinability. Analysis of variance revealed no significant difference with pollen viability at $p<.424$ among all the accessions; while though gernminabilty showed no significant difference at $p<.478$ and $p<.246$ for MS007 and MS012 respectively, the SBK differed significantly, with 0.1% treatment, at $p<.000$. The pollens were viable and possess germination ability. Optimum germination medium comprised 0.025g Boric acid. Poor seed germination in *Stevia* is unconnected with its pollen profile.

Keywords: *Stevia,* sweet glycosides, zero calorie, diabetics, seed, pollen, viability, germinability

1. Introduction

Stevia rebaudiana Bertoni is a member of the genus *Stevia* and one of only two that produce sweet steviol glycosides (Soejarto et al., 1982; 1983). It is native to subtropical and tropical South America and Central America (Robert, 2010). The leaves were used either to sweeten tea or as a general sweetening agent.

Shock (1982) reported the plant, under cultivation can reach up to 1 m or more in height. Leaves are elliptical, sessile, oppositely arranged lanceolate to oblancoelate in shape, and also deeply or slightly serrated above the middle. The flowers comprises of tiny, white, 2–6 florets which are borne in small corymbs loosely arranged in panicles. Oddone (1997) considers *Stevia* to be self-incompatible and insect pollinated.

Growing *Stevia* from seed normally has a very low germination success; sometimes only 10% (Sakaguchi & Kan, 1982). The poor seed germination problem in this plant posed a lot of obstacles towards large scale establishment of the crop and thereby making the available plant materials scarce and costly.

With the rise in percentage of diabetic human population across the World, Malaysians inclusive, the dare need to seek for a brake through that would allow *Stevia* propagation by seed becomes inevitable (Goettemoeller & Ching, 1999).

Pollens and thus pollination play fundamental role in fertilization and consequently seed and fruit sets in spermatophytes (Asma, 2008), deficiency in pollen production and performance could have direct effects on seed formation, seed viability and seed germinability.

Özbek (1993) reported that internal and external factors could limit pollen production, viability and germinability rates thus bringing about poor performance in fertilization and seed/fruit yield. High pollen quantity and quality profile therefore are central to viable seed formation and propagation in seed plants.

In *Stevia* rebaudiana there has been limited studies on the pollen grain profile, while the problem with poor seed germinability still persists. Pfahler et al. (1997) stated that studying the pollen germination and growth of pollen tube are both essential for understanding fertility problem in plants. The inability to propagate this natural sweetener crop by seeds has been a major factor limiting large scale cultivation.

In this study therefore, we investigated the pollen grain viability and germinability levels in selected accessions of *Stevia* rebaudiana bertoni.

2. Materials and Methods

2.1. Pollen Viability Studies

The accessions studied in this research include MS007, MS012 AND SOUQ BUKARI (SBK) - named after the market where it was collected in Malaysia.

Matured flowers, usually referred to as corymbs, were collected from each of these accessions. Clean dry glass slides were collected and a drop of cotton blue in lacto phenol, a stain often used for pollen viability studies (Hauser & Morrison,1964), was added unto each slide.

Pollens from these flowers were then transferred unto the stain by gently tapping the flowers at a short distance above the stain layer. Three to five flowers from same accession were used per slide in order to transfer enough pollen for microscopic observations. A clean dry cover slip was gently lowered on each stain bearing the pollens in order to avoid air trap.

The preparation was left for 30 minutes so as to allow pollen pick enough of the stain. Afterwards the slide was staged on a binocular microscope for observation at x40.

Average of 3000 pollen grains was studied. Percentage pollen viability was recorded in each case by evaluating pollen (i) staining ability and (ii) shape. Pollens which stained blue (Anderson, 1992; Nyman 1992) and possessed round shapes were considered viable. Pollens short of the above traits were recorded non viable.

Collected data were analyzed using one way analysis of variance (anova) at $p<.05$ level. Post-hoc anova was further carried out in order to partition the mean differences on case by case level.

Results were tabulated and further presented in graphical form as illustrations (Figure 1).

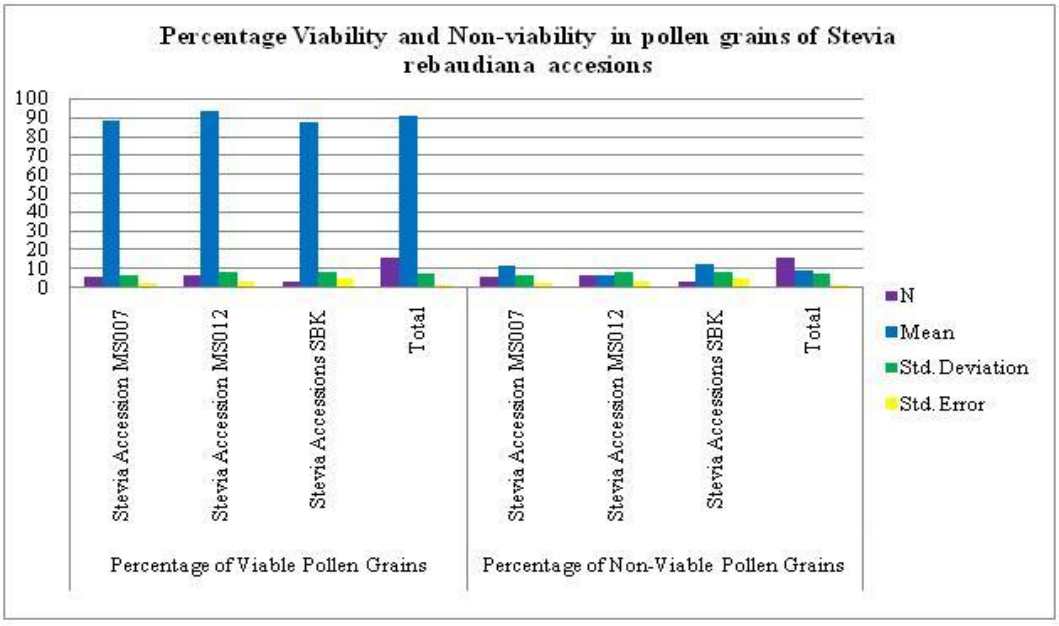

Figure 1. Showed graphical illustration of mean percentage viability & non-viability in pollen grains of accession of *Stevia rebaudiana* bertoni

2.2 Pollen Germination Studies

Carrying out this study became essential since viability may not automatically translate into germinabilty in pollen grains (Vitagliano & Viti, 1989)

Germination protocol using boric acid (0.025 g, 0.5 g and 0.1 g) added with 20 g of sucrose in 100 ml of distilled water were formulated into germination medium (GM) (Acar et al., 2010). The medium was prepared by adding and making up 20 g of sucrose in 100 ml of distilled water to produce 20% of sucrose solution then varying concentrations (grams) of Boric acid were added separately. Average of 300 pollens was scored per accession.

There were three different germination media formulated based on Boric acid varying concentrations, the first one comprised of 0.025 g, the second had 0.05 g, and the third had 0.1 g.

The mixtures were properly stirred using iron rod magnetic stirrer. Nine replicates were made of each medium which was evenly spread in Petri dishes. Collected matured flowers according to accession were then tapped above the media. For example MS007 matured flowers were tapped onto three replicates each of 0.025 g, 0.05 g and 0.1 g of boric acid concentrations. Same method was applied to other accessions.

The preparations were then incubated under 20°C for 24 hours. Germinated pollens were counted and expressed in percentages. Pollens were considered to have germinated if the length of pollen tubes were equal to, or more than, the diameter of pollen grains (Vižintin & Bohanec, 2004)

Analysis of variance and post hoc multiple comparison analysis was conducted on the collected data as explained above.

3. Results and Discussion

3.1 Pollen Viability Studies

Obtained results in this study are stated in Tables 1 and 2, and expressed graphically in Figure 1; the plates were placed in Figures 2a & b.

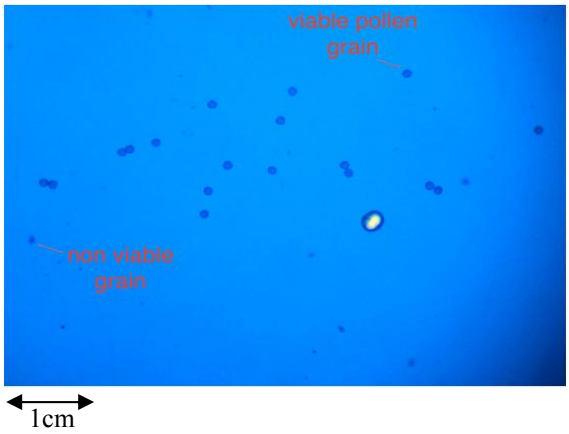

Figure 2a

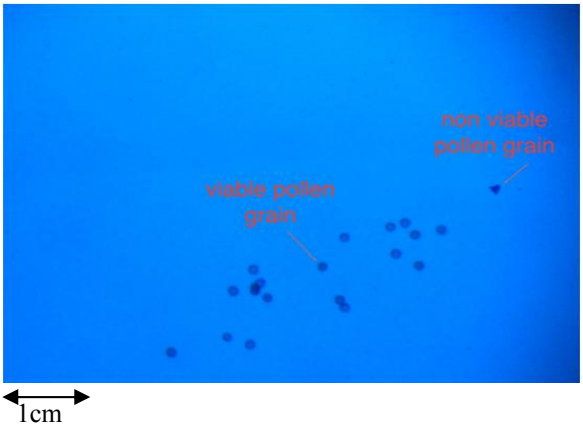

Figure 2b

Figure 2a-b. Plate showed viable & non-viable pollens of *stevia* accession in Cotton blue-in- Lactophenol

Table 1. Showed the mean percentage pollen viability in grains of accessions of *Stevia rebaudiana* bertoni

S/N	Accessions of Stevia	Mean of percentage (%) Viability	Std.Deviation
1.	MS007	88.607*	±6.46401
2.	MS012	93.3700*	±8.78873
3.	SBK	87.0567*	±8.38431

*The mean difference is not significant at the P<.05 level.

Key: MS007- Mardi Stevia 007; MS012- Mardi Stevia 012 and SBK- Souk Bukhari (accession).

Table 2. Showed results obtained on percentage of viable pollen grains from the post-hoc multiple comparisons analysis at probability level 0.05

Dependent Variable	Independent Variable (I) Types of Stevia Accesssions.	(J) Types of Stevia Accesssions.	Mean Difference (I-J)	Std. Error	Sig.
Percentage of Viable Pollen Grains					
	Stevia Accession MS007	Stevia Accession MS012	-4.76333*	4.39957	.896
		Stevia Accessions SBK	1.55000*	5.59176	1.000
	Stevia Accession MS012	Stevia Accession MS007	4.76333*	4.39957	.896
		Stevia Accessions SBK	6.31333*	5.45699	.804
	Stevia Accessions SBK	Stevia Accession MS007	-1.55000*	5.59176	1.000
		Stevia Accession MS012	-6.31333*	5.45699	.804

*The mean difference is not significant at the .05 level.

For viability in pollen grains studied across three accessions of *Stevia rebaudiana,* MS007, MS012 and SBK, the mean percentage viability (Table 1) showed all possessed high viability values with 88.607, 93. 370, and 87.057 respectively; but the MS012 had the highest value. The standard deviation values recorded for each mean of percentage viabilty revealed sample size appropriately represented their respective population sizes.

One way analysis of variance at p<.05 showed no significant difference between and within groups evalauted at p<.424. In order to observe details in the mean differences in these accessions of Sevia the post- hoc was conducted for multiple comparisons and the results shown on table 2. MS007 had lesser mean (-4.76333) compared to MS012, while SBK was lesser still in mean value compared to other two accessions with -1.55000 and -6.31333respectively. This indicated that although there are no significant differences among the accessions in viability of pollen grains, but MS012 is better off than 007 and 007 than SBK.

Figure 1 clearly showed the graphical representation of the collected data on pollen viability. It would be observed from the graph that order of increment in percentage pollen viability mean ranked as MS012>MS007>SBK. The percentage of non-viability was also in the reverse- SBK>MS007>MS012. While the 'N' represented the number of replicates, with the least being 3, the small values obtained for the standard deviations and errors in respect of the values for the mean could also be seen as expressed; a further proof that sample sizes represented the population sizes.

Viability in pollen grains is an essential condition for sexual reproduction in plants (Smith-Huerta & Vasek, 1984). The pollen grains produce two sperm nuclei each which during fertilization unite with nuclei of two other cells in the ovary thereby forming a diploid zygote and endosperm in the seed.

Viable pollen grains which could be effective for pollination are therefore necessary to form viable seeds in plants (Smith-Huerta & Vasek, 1984). The seeds are thus the means of propagation in most plants.

From the obtained results in this study, poor seed germination (Sakaguchi & Kan, 1982) observed in *Stevia rebaudiana* may not be due to the pollen grains. Perhaps other factors like seed dormancy, incompatibility (Oddone, 1997) and so on, may be responsible for the problem. Plate indicating pollen viability was represented in Figures 2a & b.

3.2 Pollen Germinabilty Studies

This study became necessary since viability in pollens alone may not guarantee the germinability (Vitagliano & Viti, 1989), thus, the quality of pollens lies in their ability to possess both traits.

Shivanna et al. (1991) reported that *In vitro* pollen germination studies were considered most appropriate means for indicating pollen germinability in plants.

Obtained results were stated on Table 3, 4, 5 & 6, while the plates and the graphical representation of the data

were placed in Figures 3a & b and 4 respectively.

Table 3. Showed the mean percentage germinability with varying boric acid concentrations in pollen grains of *stevia rebaudiana* accessions

S/N	Accessions of Stevia	Mean of percentage (%) 0.025g±SD	Germinability 0.05g±SD	(Boric acid conc.) 0.1g±SD
1.	MS007	75.6200 ±6.00	74.4200 ±10.50	65.9067 ±4.40
2.	MS012	81.7900 ±2.66	69.2133 ±7.96	65.5100 ±.95
3.	SBK	73.0133 ±13.24	62.2233 ±3.85	35.4433 ±5.22

Table 4. Showed the mean percentage germinability with 0.025g boric acid concentration in pollen grains of *stevia rebaudiana* accessions

Dependent Variabl e	Independent Variable (I) Varying Accessions of Stevia rebaudiana	(J) Varying Accessions of Stevia rebaudiana	Mean Difference (I-J)	Std. Error	Sig.
Percentage (%) of germinated Pollens with effects of Boric acid concentration 0.025g					
Bonferroni	Mardi Stevia 007	Mardi Stevia 012	-6.17000*	6.96563	1.000
		Souq Bukhari	2.60667*	6.96563	1.000
	Mardi Stevia 012	Mardi Stevia 007	6.17000*	6.96563	1.000
		Souq Bukhari	8.77667*	6.96563	.763
	Souq Bukhari	Mardi Stevia 007	-2.60667*	6.96563	1.000
		Mardi Stevia 012	-8.77667*	6.96563	.763

*The mean difference is not significant at the .05 level.

Table 5. Showed the mean percentage germinability with 0.05g boric acid concentration in pollen grains of *Stevia rebaudiana* accessions

Dependent Variable	Independent Variable (I) Varying Accessions of Stevia rebaudiana	(J) Varying Accessions of Stevia rebaudiana	Mean Difference (I-J)	Std. Error	Sig.
Percentage (%) of germinated Pollens with effects of Boric acid concentration 0.05g					
Bonferroni	Mardi Stevia 007	Mardi Stevia 012	5.20667*	6.47059	1.000
		Souq Bukhari	12.19667*	6.47059	.325
	Mardi Stevia 012	Mardi Stevia 007	- 5.20667*	6.47059	1.000
		Souq Bukhari	6.99000*	6.47059	.965
	Souq Bukhari	Mardi Stevia 007	-12.19667*	6.47059	.325
		Mardi Stevia 012	-6.99000*	6.47059	.965

*The mean difference is not significant at the .05 level.

Table 6. Showed the mean percentage germinability with 0.1 g boric acid concentration in pollen grains of *Stevia rebaudiana* accessions

	Independent Variable				
Dependent Variable	**(I) Varying Accessions of Stevia rebaudiana**	**(J) Varying Accessions of Stevia rebaudiana.**	**Mean Difference (I-J)**	**Std. Error**	**Sig.**
Percentage (%) of germinated Pollens with effects of Boric acid concentration 0.1g					
Bonferroni	Mardi Stevia 007	Mardi Stevia 012	.39667	3.25078	1.000
		Souq Bukhari	30.46333*	3.25078	.000
	Mardi Stevia 012	Mardi Stevia 007	-.39667	3.25078	1.000
		Souq Bukhari	30.06667*	3.25078	.000
	Souq Bukhari	Mardi Stevia 007	-30.46333*	3.25078	.000
		Mardi Stevia 012	-30.06667*	3.25078	.000

*The mean difference is significant at the .05 level.

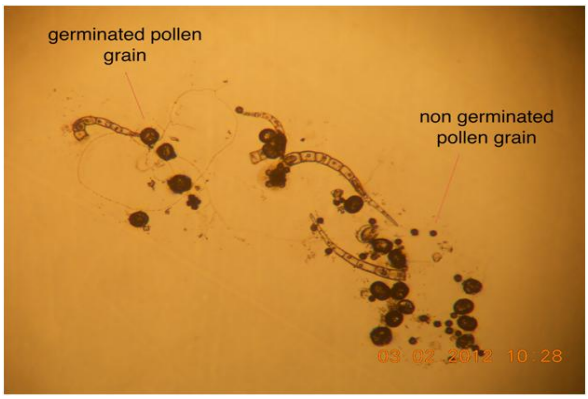

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Figure 3a

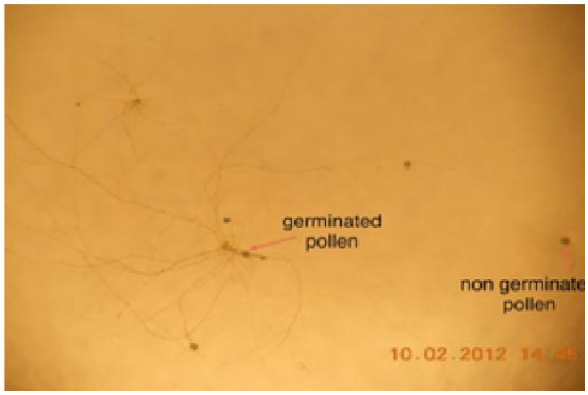

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Figure 3b

Figure 3a-b. Plate showed Viable & Non-viable Pollens of *Stevia* accession in Cotton blue-in- Lactophenol

Boric acid in varying concentration was used as the germination medium for the three accessions of Stevia, Acar et al. (2010) reported high pollen germination due to varying concentrations of Boron in form of Boric acid and sucrose solution mixture. With the 0.025 g concentration of Boric acid in sucrose solution (GM), the order of increment in mean percentage pollen germinability was SBK<MS007<MS012; for the 0.05 g germination medium, the order was SBK<MS012<MS007; while the order of increment was SBK<MS012<MS007 with the 0.1 g concentration of the germination medium.

Values obtained for the standard deviation in each accession per each treatment were not large; thereby indicating the sample sizes truly represented the population sizes accordingly.

The analysis of variance at probability level p<.05 revealed there were no significant differences on percentage of germinated pollen grains with effects of 0.025 g and 0.05 g germination media for the three accessions at p<.478 and p<.246 respectively. With the 0.1 g treatment a significant difference was observed at p<.000. A post-hoc analysis for multiple comparisons of mean was further conducted to unravel the effects of each treatment based on individual accession, in order to make a comparison there off. These are shown on Table 4, 5 and 6.

Post hoc analysis with the 0.025 g boric acid concentraton treatment showed that though statistically there was no significant difference between the three accessions on percentage of germinated pollen grains, the mean difference indicated MS007 was lessely influenced (-6.17000) as compared to the MS012.While Souq Bukhari (SBK) was lessely influenced still, -2.60667 and -8.77667 in comparison to MS007 and MS012 respectively.

Table 5 showed the values due to 0.05 g boric acid treatment effects on pollen germination in the accessions. Equally there was no significant difference, but MS012 was lessely influenced (-5.20667) when compared to MS007. SBK was lowly influenced still when compared to other two accessions MS007 and MS012 with -12.19667 and -6.99000 mean differences respectively.

Post hoc analysis with effects due to 0.1 g boric acid treatment on pollen grain germination shown on Table 6 revealed there was no significant difference between MS007 and MS012, though the latter was lessely influenced (-.39667) than than the former. SBK had signiicant difference with the parameter in question when compared to MS007 and MS012 at p<.0000 in each case, and with the respective mean differences -30.46333 and -30.06667.

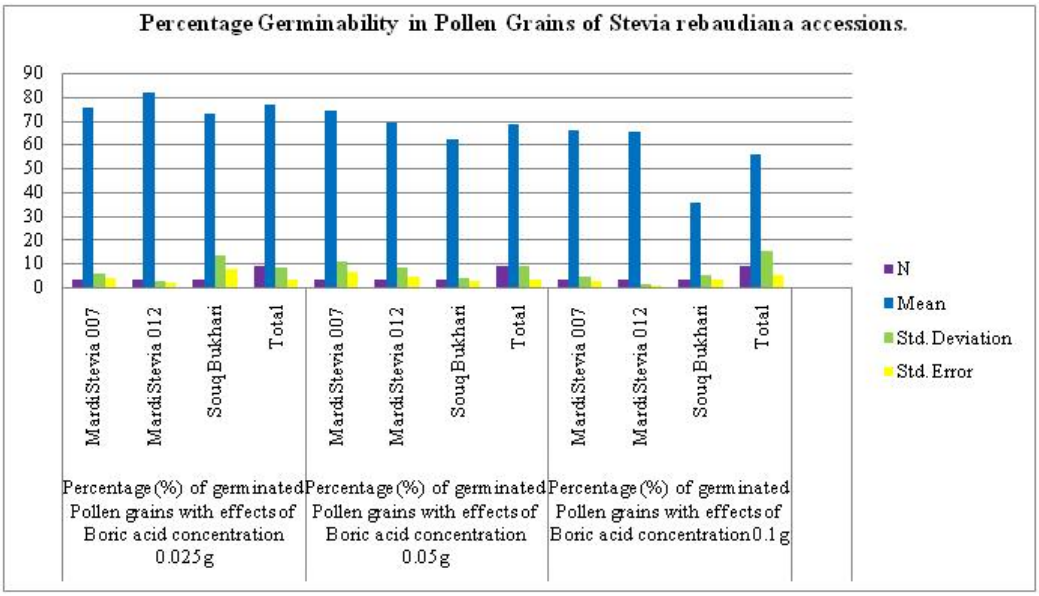

Figure 4. Showed graphical illustration of mean percentage germinability in pollen grains of accession of *Stevia rebaudiana* bertoni

Figure 4 graph expressed percentage pollen germinability across the three accessions with varying boric acid concentration treatments. It would be observed that with 0.025 g treatment MS012 expressed highest value with 81%, followed by MS007 with almost 76% and SBK with 73%. Similarly, the 0.05 g treatment showed 74% pollen germination for MS007; 69% for MS012, and 62% for SBK. Lastly with the 0.1 g treatment, variation in pollen percentage germination could be observed. MS007 and MS012 had almost equal percentage pollen germination, 65.91 and 65.51% respectively, while SBK had 35%.

The Boric acid germination media of different concentrations well supported pollen germinabilty across the accessions, though the 0.025 g seemed more effective. The observed declination (35%) in pollens of SBK treated with 0.1 g Boric acid which was at variance with its corresponding viability result (87.06%) may possibly be due to concentration effects of the acid. Previous findings have shown that pollens may be viable and still posses low

germination quality owing to environmental factors (Özbek, 1993). This result also gave an insight that the SBK collected *Stevia* accession may possibly posses different phylogenetic line from the other two.

The small values for standard deviation and standard error expressed in the graph further buttressed the fact that the sample sizes truly represented the population sizes.

4. Conclusion

The pollens in the studied *Stevia* accessions MS007, MS012 and SBK were viable and possess germination ability. Optimum germination medium for pollens in this crop comprised 0.025 g Boric acid in 20% sucrose solution. Poor seed germination problem in *Stevia* is unconnected with its pollens grains.

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