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Dry Leaf Biomass Stability of Stevia (*Stevia rebaudiana*) Clones over Different Environments

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

In order to identify stable high-yielding stevia (*Stevia rebaudiana*) genotypes, a multi-environment testing was conducted over four different growing environments. The experiments were conducted in a randomized block design, with three replications during 2019 and 2020. The combined analysis of variance showed significant variation for genotype, environment, and G×E interaction for all studied traits. The highest mean performance for all the traits revealed that Hoshiarpur has favorable conditions for stevia cultivation, and CSIR-IHBT-ST-1801 followed by CSIR-IHBT-ST-G12 were the best performers over all the locations. The Eberhart and Russell model-based stability parameters demonstrated that CSIR-IHBT-ST-1801 was a stable performer for dry leaf biomass, and that was also further confirmed by GGE biplot analysis. Primary shoots were major contributors

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to the dry leaf biomass, as indicated by the substantial positive leaf biomass contribution shown by Pearson's correlation coefficients. As a result, primary shoots might be utilized as selection criteria to increase the dry leaf biomass. The CSIR-IHBT-ST-1801 could be used as a stable high-yielding variety for the targeted regions and also, can be used for further stevia breeding programs.

Keywords: Stevia; multi-environment; dry leaf biomass; stability; G×E interaction.

1. INTRODUCTION

The South American native perennial herb stevia (*Stevia rebaudiana*) has garnered a lot of interest lately due to its remarkable sweetness and possible health benefits. This plant, belonging to the Asteraceae family, is generally referred to as "stevia." It is typically grown for its leaves, which are collected for their naturally occurring steviol glycosides. Traditional bitter beverages like mate tea have been sweetened using dry stevia leaves [1].

stevia has been used in food products as a natural, low-calorie sweetener in place of sucrose. Beyond being used to sweeten food and beverages, stevia extracts are also used in a variety of medications, cosmetics, and dental care items. It has biological properties that include antileukotriene, anti-cancer, insulin secretagogues and reduces the effect of heart diseases [2]. It plays a significant role in the food business, due to the presence of sweet-tasting diterpenic glycosides (SGs) in its leaves, primarily stevioside (Stv) and rebaudioside A (Reb A) [3,4]. There are further reports of traces of rebaudioside B, D, E, and F as well as minor compounds [3,5,6] such as rebaudioside C (Reb C) and duloside A (Dul A) [5,7].

These glycosides, particularly stevioside and rebaudioside A, are responsible for the exceptional sweetness of stevia, which can be up to 300-450 times sweeter than traditional table sugar [8,9], whereas Zhang and Bell reported that this specie is 200-300 times sweetener then sucrose [10,11]. Even with its exceptional sweetness, stevia is practically calorie-free and has no effect on blood sugar levels, which makes it a desirable substitute for people trying to cut back on sugar or who are managing health issues like diabetes and obesity [10,12].

Researchers use the GGE (Genotype Main Effect plus Genotype-by-Environment Interaction) biplot, a potent tool for analysing multi-environment trial data and evaluating genotype performance in various contexts. The GGE biplot approach on *Stevia rebaudiana*, can give important insights into the impact of environmental factors (growing conditions, locations, etc.) and genotype (various cultivars or variations of stevia) on the plant's growth, yield, and quality parameters [10]. Breeders can use the GGE biplot to guide selection tactics for crop development and cultivation techniques by identifying stable genotypes and particular genotype-by-environment interactions. The Eberhart and Russel model was used to understand how different genotypes would have reacted to different environmental conditions. This will help in the development of improved cultivars that display desirable traits like high dry leaf biomass and adaptability to different growing regions. Hence, the current study was undertaken to assessed the stevia clones for stable and higher dry leaf biomass over multi-environments using.

2. MATERIALS AND METHODS

2.1 Planting Material and Experimental Layout

A set of eight clonal lines of stevia (*Stevia rebaudiana*) along with a check (Him Stevia) (Table 1) were evaluated for dry leaf biomass and associated traits. Experiments were laid out in randomized block design that were replicated thrice at four different locations ie., Palampur, Ludhiana, Hoshiarpur, and Kichha. For that twenty-five days old plants of each genotype were transplanted in a field by keeping 45×45 cm spacing (50 plants per plot) at each location in 2017. Weeding was scheduled to be done every 20 days during the first two months, to prevent crop weed competition and ensure weed-free plots everywhere. Also, to ensure good crop growth at all sites, regular irrigations were applied. Farmyard manure that had roten down well was applied at a rate of 20 t/ha before transplantation as part of the field preparation. Additionally, 100:40:50 kg/ha NPK was applied for higher dry leaf biomass, the nitrozen split in four doses and broadcast every year, while potash and phosphorous were applied once as basal dose. All additional recommended agronomic practices were applied to ensure good crop growth at each site.

Table 1. Details of planting material used in the study

Sr. no.	Genotype
1	CSIR-IHBT-ST-G1
2	CSIR-IHBT-ST-G12
3	CSIR-IHBT- ST-1801
4	CSIR-IHBT-ST-G27
5	CSIR-IHBT-ST-G142
6	CSIR-IHBT- ST-G290
7	CSIR-IHBT- ST-27
8	Him Stevia
9	CSIR-IHBT- ST-41

Table 2. Details of different locations, their average annual weather parameters, and soil type

Sr.no.	Parameters	Palampur	Ludhiana	Hoshiarpur	Kichha
1.	Altitude (m)	1300	244	296	293
2.	Average minimum temperature (°C)	11.83	12	14	20.49
3.	Average maximum temperature (°C)	21.70	40.5	41	31.97
4.	Total rainfall (mm)	1578	733	122.93	91.68
5.	Relative humidity (%)	62.58	68	37	46.09
6.	Soil texture	Silty clay loam	Clay loam	Sandy loam	Stiff clay
7.	Soil pH	5.23 - 6.10	7.8-8.5	6-8.2	8.0-8.2
8.	Soil type	Acidic	Alkaline	Acidic	Acidic

2.2 Experimental Site

The details of locations with their weather parameters are given in Table 2. The experimental sites lay from 244 to 1300 m above mean sea level. The physiochemical properties of soil varied from acidic silty clay loam to alkaline sandy loam.

2.3 Data Observation

The data on morphological traits were recorded for the two years during 2019 and 2020 from second year onwards after transplanting. For that, five plants from each plot were randomly selected for data observation on the number of primary shoots, Leaf-stem ratio, leaf length (cm), leaf width (cm) and dry leaf biomass (g/plant). The dry leaf biomass was observed by harvesting the leaves from the whole plant and drying in shade.

2.4 Statistical Analysis

Before doing a parametric stability analysis, the entire data set was first examined for homogeneity of variance across the locations and years using the "Bartlett test" function in RStudio [13]. OPSTAT was used to compute the stability analysis using the Eberhart and Russell model, 1966 [14] and the combined analysis of variance using a two-factor ANOVA [15]. The "geplot" function in RStudio was used to create the heatmaps representing the average genotype performances throughout the locations. The "GGE" and “correlation” functions in RStudio were used to perform analyses for the GGE biplot and Pearson's correlation.

3. RESULTS AND DISCUSSION

3.1 Combined Analysis of Variance

The Bartlett test was used to verify the homogeneity of variance across the years and locations (Table 3). The variances mentioned are homogeneous, as evidenced by the non-significant ($P < 0.05$) K-squared values for all the traits analysed across all locations and years. The treatment means pooled for additional analysis over the years due to the homogenous variance over the two years. The assumptions of the parametric analysis of stability using the Eberhart and Russell model are satisfied by the observed homogeneous variance over the locations. The combined data from both years was subjected to a combined analysis of variance for all the assessed traits once the homogeneity of variances was validated. This allowed for the partitioning of the entire variance in genotype (G), environment (E), and G×E interactions, and their testing against the residual (Table 4). All the traits were highly significant due to the main effects of genotype and environment (Table 4). The findings further illustrated the strong effects of the environment by demonstrating the high genetic variation of these traits. Similar results were revealed in twenty genotypes of celery, the all-seed yield-related traits were highly significant in genotypes, locations, years, and interactions based on tested G×E interaction [16].

Different Genotypes responded differently in different environments, as indicated by the G×E interaction and predicted that, stevia genotype testing at several locations to facilitate selection and effectively release new varieties. A similar result was observed by Kumar et al., 2020 and they found a comparable degree of diversity in genotypes and environments with a significant G×E interaction after evaluating sixteen genotypes including one check (AWS-1) of ashwagandha in Agroclimatic Zone-IV of Gujarat [17]. This noteworthy combined analysis of variance results permitted additional stability analysis to be carried out for key economic characteristics, such as dry leaf biomass.

3.2 Performance per se

Mean comparisons of all the traits showed that Hoshiarpur (Punjab) has higher mean performances than the overall mean for primary shoots, Leaf-stem ratio, and dry leaf biomass, whereas Palampur (Himachal Pradesh) showed significant variations in primary shoots, leaf length, and leaf width (Table 5). Genotypes CSIR-IHBT-ST-G1, CSIR-IHBT-ST-G12, CSIR-IHBT-ST-1801 and Him Stevia showed higher mean performances than the overall mean for primary shoots trait, whereas CSIR-IHBT-ST-G1, CSIR-IHBT-ST-G27, CSIR-IHBT-ST-27 and Him Stevia showed higher mean performance than overall mean for the Leaf-stem ratio trait. However, CSIR-IHBT-ST-G1, CSIR-IHBT-ST-1801, and CSIR-IHBT-ST-27 genotypes had higher mean than overall mean for leaf length and CSIR-IHBT-ST-G1, CSIR-IHBT-ST-G12, CSIR-IHBT-ST-1801, CSIR-IHBT-ST-27 and CSIR-IHBT-ST-41 genotypes had a higher mean than overall mean for leaf width. Genotypes CSIR-IHBT-ST-1801, CSIR-IHBT-ST-G12, and Him Stevia showed higher mean performances than the overall mean for dry leaf biomass.

Table 3. Test of homogeneity of variance over the locations and years

Traits	Over the locations			Over the years		
	Degree of freedom	Bartlett's Ksquared	p-value	Degree of freedom	Bartlett's Ksquared	p-value
Primary shoots	3	2.27^{NS}	0.52	1	0.00^{NS}	0.95
Leaf-stem ratio	3	40.72^{NS}	7.5e-9	1	0.20^{NS}	0.65
Leaf length	3	5.17^{NS}	0.16	1	2.09^{NS}	0.15
Leaf width	3	10.80^{NS}	0.01	1	1.07^{NS}	0.30
Dry leaf biomass	3	1.04^{NS}	0.79	1	0.09^{NS}	0.77

^{NS} non-significant at $p \leq 0.05$

Table 4. Combined analysis of variance for all the traits pooled over both years

Source of Variation	Degree of freedom	Mean sum of squares				
		Primary shoots	Leaf-stem ratio	Leaf length	Leaf width	Dry leaf biomass
Replication	2	7.85	5.44	0.87	0.15	6.32
Genotype (G)	3	60.61^{**}	14.39^{**}	2.68^{**}	1.69^{**}	4.39^{**}
Environment (E)	8	121.61^{**}	4.62^{**}	1.61^{*}	0.50^{*}	1309.40^{**}
G x E	24	9.97^{**}	4.84^{**}	2.43^{**}	0.70^{**}	10.33^{**}
Error	70	3.39	1.58	0.76	0.22	0.63

*,** significant at $p \leq 0.05$ and $p \leq 0.01$, respectively

Table 5. Genotype's and test location's mean comparisons for all the traits pooled over the years

Genotypes/ Environments	Primary shoots	Leaf-stem ratio	Leaf length (cm)	Leaf width (cm)	Dry leaf biomass(g/plant)
CSIR-IHBT-ST-G1	11.32^{c*}	6.55^{cd*}	6.54^{bc*}	3.46^{b*}	75.39^{d}
CSIR-IHBT-ST-G12	14.27^{d*}	5.43^{ab}	6.05^{ab}	3.16^{ab*}	85.05^{f*}
CSIR-IHBT-ST-1801	16.37^{e*}	5.24^{a}	6.72^{bc*}	3.33^{b*}	95.31^{g*}
CSIR-IHBT-ST-G27	9.47^{b}	6.79^{d*}	6.08^{ab}	2.87^{a}	63.62^{a}
CSIR-IHBT-ST-G142	8.87^{b}	5.61^{abc}	5.86^{a}	2.92^{a}	64.15^{a}
CSIR-IHBT-ST-G290	8.72^{b}	5.76^{abc}	6.00^{a}	3.09^{ab}	67.30^{b}
CSIR-IHBT-ST-27	7.08^{a}	6.43^{bcd*}	6.89^{c*}	3.23^{ab*}	75.51^{d}
Him Stevia	14.70^{d*}	6.96^{d*}	6.04^{ab}	2.92^{a}	81.93^{e*}
CSIR-IHBT-ST-41	10.16^{bc}	5.98^{abcd}	6.06^{ab}	3.23^{ab*}	72.20^{c}
C.D.	1.50	1.03	0.71	0.38	0.65
Palampur	12.24^{C*}	5.21^{A}	6.64^{B*}	3.43^{C*}	75.43^{A*}
Ludhiana	11.07^{B}	5.91^{B}	6.11^{A}	3.20^{BC*}	75.12^{A}
Hoshiarpur	12.40^{C*}	6.96^{C*}	5.90^{A}	2.83^{A}	75.95^{B*}
Kichha	9.15^{A}	6.26^{B*}	6.35^{AB*}	3.08^{B}	75.92^{B*}
C.D.	1.00	0.68	0.48	0.25	0.43
Overall mean	11.22	6.08	6.25	3.13	75.61
SE(d)	0.75	0.51	0.36	0.19	0.32
SE(m)	0.53	0.36	0.25	0.14	0.23

*Different superscript letters and numbers show significant ($p \le 0.05$) difference between the means within the column for genotypes and environments, respectively; *Significantly ($p \le 0.05$) higher than the overall population mean; CD- critical difference*

Table 6. Stability parameters for dry leaf biomass based on Eberhart and Russell model

Genotypes	Dry leaf biomass (g/plant)			
	Mean	b_i	S^2d_i	**R**
CSIR-IHBT-ST-G1	75.39	1.44	1.40	F
CSIR-IHBT-ST-G12	85.05*	1.66	1.94	F
CSIR-IHBT-ST-1801	95.31*	1.02	-0.20	S
CSIR-IHBT-ST-G27	63.62	4.03	-0.90	F
CSIR-IHBT-ST-G142	64.15	4.78	-1.52	F
CSIR-IHBT-ST-G290	67.30	-0.67	0.45	U
CSIR-IHBT-ST-27	75.51	1.44	1.69	F
Him Stevia	81.93*	1.33	1.93	F
CSIR-IHBT-ST-41	72.20	0.94	-1.34	S

**, higher than the overall mean; b_i , regression coefficient; S^2d_i , deviation; R, stability responses as (F, suitable for favorable environment; U, suitable for un-favorable environment and S, stable)*

Moreover, CSIR-IHBT-ST-1801 had a significantly higher number of primary shoots (16.37 primary shoots), leaf length (6.72 cm), leaf width (3.33 cm), and dry leaf biomass/ plant [95.31 gram (5.0 tons/ha)] than the overall mean at four locations as compared to check (Him Stevia) and other stevia genotypes.

The environment-2 exhibits favorable conditions for German chamomile growing, as indicated by the highest mean performance for most attributes [18]. Among all the locations, MC19005 demonstrated the best performance [18]. Similarly, present findings provided evidence that CSIR-IHBT-ST-1801 would be a better option for improving all component qualities at once in any plant breeding program. However, CSIR-IHBT-ST-1801's highest mean values for the economic trait, i.e., dry leaf biomass, showed that it could be grown commercially.

3.3 Stability Analysis

3.3.1 Regression-based Eberhart and Russel model

To select and develop new varieties of stevia, the most significant and economically desired trait is the dry leaf biomass. As a result, various Eberhart and Russell model stability factors related to dry leaf biomass were examined and are shown in Table 6. Three metrics, such as the mean, regression coefficient, and deviation from the regression coefficient, were taken into consideration to assess stability. Similar three metrics were utilized to evaluate the stability of ninety-six genotypes of ashwagandha [19].

For dry leaf biomass, the regression coefficients for several genotypes varied from -0.67 to 4.78. Out of nine genotypes, three genotypes named CSIR-IHBT-ST-G12, CSIR-IHBT-ST-1801, and Him Stevia showed a higher mean than the overall mean. Genotype CSIR-IHBT-ST-1801 had unity regression coefficients with minimum deviation from the regression and it also had a higher mean than the overall mean. However, another genotype named CSIR-IHBT-ST-41 showed a near unity regression coefficient but its mean was lesser than the overall mean. Consequently, different environments have demonstrated consistent responses from these two genotypes, CSIR-IHBT-ST-1801 and CSIR-IHBT-ST-41. The CSIR-IHBT-ST-G290 genotype was shown to be suited for unfavorable environmental conditions due to its smallest divergence from the regression coefficient and regression coefficient smaller than unity. Whereas, the regression coefficients greater than one for the genotypes CSIR-IHBT-ST-G1, CSIR-IHBT-ST-G12, CSIR-IHBT-ST-G27, CSIR-IHBT-ST-G142, CSIR-IHBT-ST-27, and Him Stevia indicated that these genotypes were appropriate for favorable environmental conditions.

Overall, the findings demonstrated that CSIR-IHBT-ST-1801 performed better on average than the overall mean and responded steadily to various environments; as a result, it may be encouraged for commercial cultivation of that genotype in various environmental conditions for the production of dry leaf biomass. Commercial cultivation across environments should prioritize genotypes with high mean values, regression coefficients equal to unity, and minimal deviations equal to zero for desired traits.

3.3.2 Heatmap and GGE biplot

The purpose of creating the heat map visualizations was to understand how each genotype performed with the others at every tested location. The heatmap of dry leaf biomass showed mean performances of all the genotypes in four different environmental conditions. The genotype CSIR-IHBT-ST-1801 at Ludhiana had the highest dry leaf biomass, whereas genotype CSIR-IHBT-ST-G27 at Palampur had the lowest dry leaf biomass. Findings indicated that the relationship between genotype and environment affected the dry leaf biomass. Overall, the heatmap showed that genotype CSIR-IHBT-ST-1801 (95.3 g/plant) had higher dry leaf biomass followed by CSIR-IHBT-ST-G12 (85 g/plant) and Him Stevia (81.9 g/plant). Future breeding initiatives may use these genotypes to improve dry leaf biomass that is specific to tested locations. The heatmap also depicted that Hoshiarpur and Kichha locations (75.9 g/plant each) were best suited for higher dry leaf biomass followed by Palampur (75.4 g/plant) and Ludhiana (75.1 g/plant).

The top performing genotype in the target environment was determined using GGE biplot analysis based on which-won-where patterns of genotype mean performances and stability across the multi-environments. The total variability of 99.17% (98.12% and 1.05%, respectively) was supplied by the first two principal components (PC1 and PC2) of the GGE biplot (Fig. 1). To determine the relationship between genotypes and locations as well as the existence of mega environments, the polygon and vertices of GGE biplots were utilized. The genotypes CSIR-IHBT-ST-1801, CSIR-IHBT-ST-G12 and Him Stevia were located at the polygon's vertices in terms of dry leaf biomass. The biplot is divided into four enormous environmental regions by the perpendicular rays that are drawn from its origin to each side of the polygon. Among those environmental sectors, all genotypes at the polygon's corner are thought to be the greatest performers. One sector encompasses all of the studied locations, and genotype CSIR-IHBT-ST-1801 outperformed others in this sector. It showed that the combined effects of all the test sites created a massive habitat for dry leaf biomass. The results showed that, throughout the studied conditions, genotype CSIR-IHBT-ST-1801 performed the best, and exhibited constant expression.

Similar results were found by Mustamu et al., 2018 on sweet potato genotypes, and GGE Biplot research predicted that stable genotypes were Awachy1, 80 (109) and 68 (120) Ayamurasaki [20]. The Eberhart and Russell regression model, which takes into account all test locations, predicts that CSIR-IHBT-ST-1801 was the top performer in all tested locations based on the GGE biplot results. Similar GGE biplot analysis investigations have been conducted in different crops, focusing on grain yield and quality traits [16,21-23].

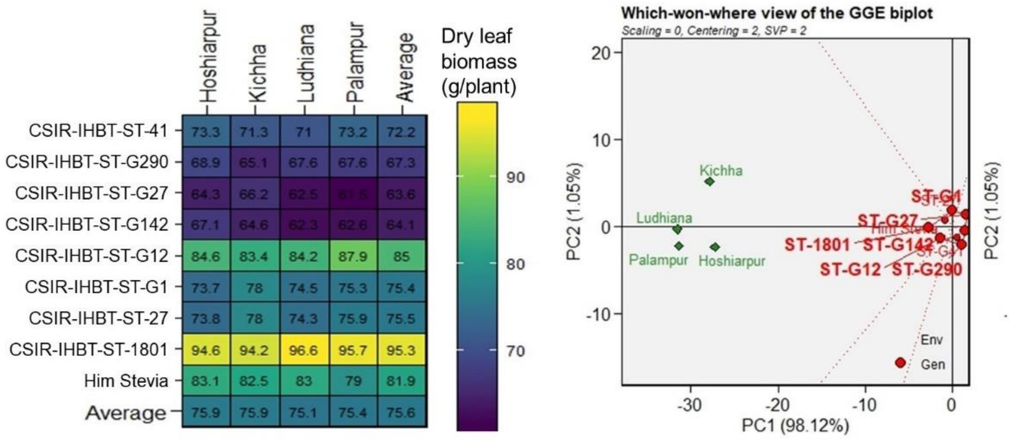

Fig. 1. Heatmap depicted mean performances of the genotypes at all tested locations for dry leaf biomass (left), and "Which-won-where" type GGE biplots for dry leaf biomass representing the genotype main effect plus G×E interaction (right)

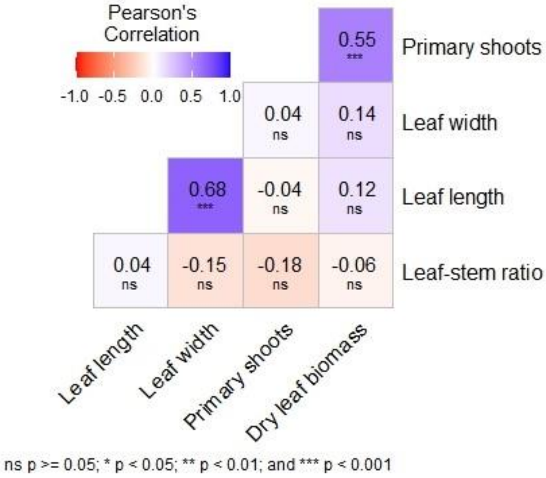

Fig. 2. Pearson's correlation coefficients among all the traits

In earlier reports, seven regions of the three tested locations were represented by the GGE biplot-based which-won-where graph, each with a distinct high-yielding genotype of stevia [10]. The most prevalent and productive genotypes in Garut were G23, G12 in Sumedang, and G15 in Bandung [10]. The sweet potato genotype showed a higher yield, which was situated at the top of the region [20,24]. Similar results were found in the proso millet GGE biplot-based which-won-where graph, that the genotypes situated at the top region were more adaptive in different environments than other genotypes [25]. Contrasting results were found in maize, some genotypes situated at the center of the region, which showed less genotype and environmental effect and was found stable [26]. In a similar frame, certain stevia genotypes located in the middle region had the lowest GEI effects but low yields [10]. Similarly, genotype CSIR-IHBT-ST-1801 is situated center of the region and was found stable with high dry leaf biomass. Vaezi and coworkers suggested that additional stability parameters are needed to select the stable and high-yield barley genotypes [27,28].

3.4 Dry Leaf Biomass Contributing Traits

Leaf width showed a positive and highly significant association with leaf length (0.68), the related investigation of dry leaf biomass with other parameters revealed the presence of a high magnitude of positive and significant correlation with primary shoots (0.55). The primary branches and number of umbels per plant showed a significant correlation with celery seed yields. Thus, it was suggested that all of these features may be improved at the same time based on a positive correlation between seed yield and the number of umbels per plant and primary branches [16]. Similarly, primary shoots and dry leaf biomass positively correlated with each other, so the features that contribute to the dry leaf biomass in stevia are the primary shoots. Similarly, Enyew et al., 2021 revealed that positive correlations were found between panicle weight and sorghum grain yield, indicating that parallel selection of two traits is required for successful selection [21]. Similar to Enyew's study, the current studies have shown that effective selection necessitates the parallel selection of dry leaf biomass and primary shoots.

4. CONCLUSION

The study aimed to find superior and stable genotypes for dry leaf biomass over different environmental conditions. The combined analysis of variance revealed sufficient variability by genotypes, environments, and their interactions. The CSIR-IHBT-ST-1801 was the best-performing genotype. Furthermore, the Hoshiarpur and Kichha were the most suited locations for stevia cultivation, followed by Palampur and Ludhiana. According to the GGE biplot analysis and the stability parameters of the Eberhart and Russell model, CSIR-IHBT-ST-1801 is a stable and superior genotype for dry leaf biomass. GGE biplot analysis further demonstrated that every tested location displayed a single mega-environment. Based on the strong positive correlation coefficients between dry leaf biomass and number of primary shoots, it was determined that the number of primary shoots was the main contributor to the dry leaf biomass. It could be possible to increase dry leaf biomass by choosing a greater number of primary shoots. The current study's findings offer a solid foundation for the identification of superior and stable genotypes of stevia.

5. SYNOPSIS

- CSIR-IHBT-ST-1801 could be used as a stable high-dry leaf biomass variety for the targeted regions and also, can be used for further stevia breeding programs.
- Primary shoots were major contributors to the dry leaf biomass, so primary shoots might be utilized as selection criteria to increase the dry leaf biomass.
- Hoshiarpur and Kichha were the most suited locations for stevia cultivation.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during the writing or editing of manuscripts.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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