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# Genetic variability analysis in elite black pepper genotypes using morpho-physiological and yield-attributing traits

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## Abstract

The genetic variability in selected 21 black pepper accessions was analyzed based on desirable drought-tolerant and susceptible characteristics using principal component and cluster analyses. The experiment was conducted at ICAR-Indian Institute of Spices Research, Experimental farm, Peruvannamuzhi, Kozhikode using a randomized block design with four replications. Morphological, physiological and yield contributing traits were studied. The traits examined showed a comprehensive range of variability. The principal component and UPGMA clustering analyses were employed to assess the proportional contribution of various traits and grouped the genotypes, respectively. The first principal component was responsible for the highest variation (30.87%) in the yield-related characteristics, which were positively correlated with each other and correlated negatively with the morphological characteristics and stomatal frequency. Separate clusters were formed for the genotypes that displayed drought-tolerant characteristics (cluster 2 and 3) and those that showed susceptible characteristics (cluster 1). The results indicated that the analysed black pepper genotypes have significant genetic variability among them which may be helpful for identification of genotypes with desirable drought tolerant characteristics. Accessions 7211 (cluster 2), 1495, 1343 and 4132 (cluster 3) showed characteristics that make them potentially drought tolerant while the accessions 5717 and 4064 (cluster 1) showed drought susceptible traits.

**Keywords:** Drought tolerance, cluster analysis, principal components, pearson correlation, stomatal frequency

## Introduction

Drought emerges as a primary environmental constraint for black pepper (*Piper nigrum* L.) production, especially considering its cultivation as a rain-fed crop in the southern part of India (Krishnamurthy *et al*., 2016). Despite its widespread cultivation in countries like Vietnam, Malaysia, and Indonesia (Ambrozim *et al*., 2022), black pepper remains sensitive to drought, requiring between 2,000 and 3,000 mm of water during the reproductive stage (Yudiyanto *et al*., 2014).

In response to drought stress, black pepper plants undergo morphological, physiological, and biochemical changes to adapt to water-scarce conditions, as reported by Krishnamurthy *et al*. (2000). Traits such as high leaf area, elevated stomatal density, high stomatal conductivity, low wax content, and lower root growth make pepper plants susceptible to water stress due to increased water loss (Suleiman *et al*., 2021).

Drought-tolerant black pepper genotypes exhibit increased leaf wax content as a response to water scarcity, serving as a means of conserving water to survive in arid environments, with the cuticle playing a role in limiting water evaporation from the leaves (Thankamani and Ashokan, 2002). Futhermore, plants have developed various mechanisms to tolerate drought encompassing deeper root systems, a higher root-shoot ratio, reduced transpiration and photosynthesis, proline accumulation, ABA accumulation, inhibition of chlorophyll degradation, and balanced water status and ionic distribution, as well as carbon distribution and consumption (Kanavi *et al*., 2020; Pinheiro *et al*., 2005).

In the present study, we tried to identify drought tolerant black pepper genotypes from a group of germplasm accessions based on some assumptions using principal component analysis (PCA), pearson correlation analysis and cluster analysis, elucidating the correlation between drought tolerance characteristics and the crop drought tolerance while avoiding the bias of a single indicator (Huseynova *et al*., 2007). Numerous drought-resistant indicators are challenging to take into account for screening purposes. PCA and cluster analysis are considered among the most crucial multivariate analysis techniques (Oyelola *et al*., 2004). Additionally, cluster analysis can be employed to evaluate genetic similarity and dissimilarity in datasets by grouping the genotypes based on the characteristics under study. The implementation of PCA and cluster analysis together will improve the accuracy and usefulness of the screening of crop varieties for stress response. The objective of the current study was to identify black pepper genotypes with drought-tolerant characteristics using dendrogram-based cluster analysis and PCA.

## Materials and methods

### Plant material

Twentyone black pepper genotypes were selected based on drought tolerance response. The experiment was conducted in a randomised block design with four replications at the Peruvannamuzhi experimental farm of ICAR- Indian Institute of Spices Research, Kozhikode during 2021-22. Seventeen quantitative traits were monitored and recorded, which includes leaf length (LL), leaf width (LW), leaf area (LA), petiole length (PL), internodal length (IL), wax content (WC), number of stomata (NS), number of spikes plant$^{-1}$ (NSP), spike length (SL), peduncle length (PL), berry size (BS), number of matured berries spike$^{-1}$ (NMB), number of immature berries spike$^{-1}$ (NIB), test weight (TW), 100 berry fresh weight (BFW), 10 spiked berries weight (SBW) and 10 rachis weight (RW).

**Analysis of morphological characteristics**

To determine the leaf area, four replicates of two random leaves were collected from each accession. The length and width of each leaf were measured using a centimetre ruler, and the leaf area was determined as a product of length X width and a constant (0.71) as per Mohankumar and Prabhakaran (1980). The length of the petiole was assessed by measuring the distance starting from the leaf base and ending at the point where the petiole is connected to the stem. In a comparable manner, the length of both the petiole and internode were gauged with similar number of replications for each accession. A ruler marked with centimetre increments was used to determine all of the measurements of length and width in this study.

**Analysis of yield related characteristics**

The dimensions of five black pepper matured berries from each variety (in three replications) were determined by using a Vernier caliper with a precision of ±0.01 mm. The measurements were taken along both the axial and transverse axes and the berry size was determined as follows: Berry size (mm) = main scale division + (Vernier scale division × least count)

Other morphometric measurements of yield related traits (NSP, TW, SL, PL, NMB, NIB, BFW, SBW and RW) were collected from four replicates of each accession, with the mean of two readings for each replication.

**Determination of leaf stomatal density**

In order to take stomatal impressions, a viscous solution was prepared by dissolving thermocol in xylene. The prepared liquid suspension was layered uniformly on the abaxial and adaxial surfaces at the center of each leaf. The dried coat of viscous solution was gently peeled off from the leaf after 10-15 minutes. Then the transparent layer was mounted on a clean glass slide and a cover slip was placed over it. The prepared glass slide was viewed under the compound microscope, LEICA, Wetzlar, Germany. Stomatal density (number per $\mu m^2$) was counted from three microscopic fields chosen at random from four replications for each genotype, using 10X magnification with an image size of 391.634 μm x 522.517 μm (Fig. 1).

**Determination of leaf wax content**

The colorimetric approach developed by Blum and Ebercon (1976) was used to quantify the epicuticular wax load. The leaf area (4.84 $cm^2$) was cut from the centre portion of the leaf, excluding the midrib, for the wax extraction. The leaf pieces were immersed one at a time, each for 10 s, in 5 ml chloroform in a 15 ml beaker. The solvent was concentrated by evaporating it in a water bath set at $70^0$ C until it became dry. Then, 5 ml of wax reagent was added to this dried content and boiled in a steaming water bath for 30 minutes, cooled and then 12 ml of deionized water was added. The filtrate was collected and the intensity of colour was read at 590 nm using Shimadzu UV-Visible Spectrophotometer (UV-1800). The amount of wax in the leaves of each genotype was carefully evaluated using four different sample sets to ensure the precision.

Wax reagent: Powdered potassium dichromate (20 g) and 40 ml of distilled water were blended to create a slurry, which was then mixed with 1 litre of strong sulfuric acid to prepare wax reagent. A clear solution was prepared by heating the resultant slurry. A wax standard graph was developed by using carnauba wax.

**Statistical analysis**

Principal component analysis (PCA) was applied to the correlation matrix of the seventeen variables and twentyone genotypes, in order to find the parameters that best reflect the tolerance to response variables. Past 4.03 software was used for the analysis of principal components, Eigen values, Eigen vectors and 2D biplot visualization of PC1 and PC2. Pearson coefficient analysis was used to assess the strength of the correlation between these parameters. UPGMA cluster analysis was used for grouping the genotypes into clusters using R- software.

**Results and discussion**

Morpho-physiological and yield attributing characters studied revealed genetic diversity among the genotypes, which was explained by the findings of PCA. As per this criterion, the first five components in the current study were responsible for 73.88% of total variation for the quantitative traits (Table 1). PCA revealed seventeen components, out of which five principal components (PCs) showed eigenvalues greater than one, suggesting a significant impact on selected accessions. Reshma *et al*. (2022) reported a similar result among the black pepper genotypes in their quantitative traits with significant diversity accounted for by the six primary components. Considerable variation in the performance of black pepper genotypes, which were located in lowland and high-altitude areas arise from the changes in both genetic composition and environmental influences (Sainamole *et al*., 2002). Genetic variability and environmental factors led to the significant variation in the selected black pepper genotypes for the observed quantitative traits in the current study.

The first principal component accounted for the highest variability of 30.87% with substantial loadings recorded for spike length (0.753), 10 spiked berries weight (0.693), 10 rachis weight (0.623) and number of spikes plant$^{-1}$ (0.592), which contributed in a positive direction while the leaf area (-0.742), leaf width (-0.705), leaf length (-0.587), number of stomata (-0.530) and petiole length (-0.414) contributed negatively to PC1 (Fig. 2). The yield attributing characters and wax content mainly contributed to the highest variability in PC1. Bhor *et al.* (2021) also reported that traits associated with yield displayed the highest level of variability. Yield related contribution had a great impact on variation captured by PC1 than on the variation explained by remaining principal components (Reshma *et al.*, 2022). It is evident that PC1 experienced the highest variance and was followed by others, as reported in all the studies that employed principal component analysis.

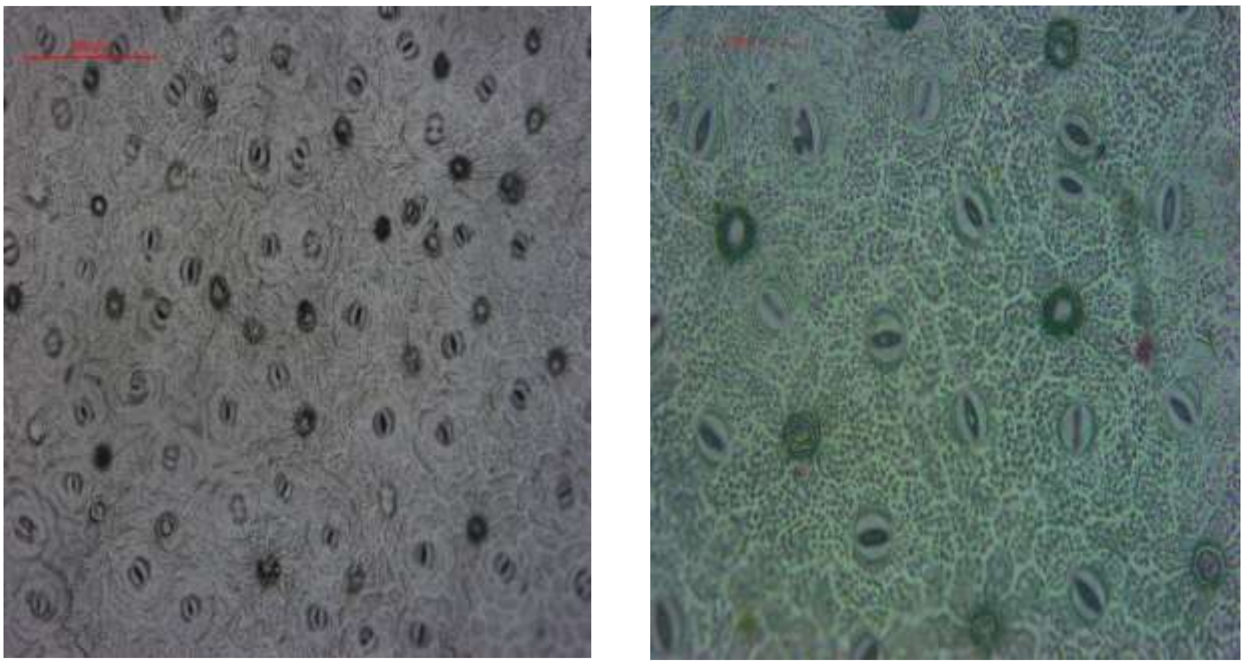

**Fig. 1.** Stomata on the ventral side of black pepper leaves (10X and 20X, image size: 391.634x 522.517 μm).

The second component accounted for 14.90% of total variation, contributed positively by number of matured berries spike$^{-1}$ (0.752), test weight (0.730) and internodal length (0.355). The remaining traits *viz.* 100 berry fresh weight (-0.654) and berry size (-0.648) contributed in the negative direction. Characteristics that constitute the yield components of black pepper genotypes were positively correlated with the actual yield (Shivakumar *et al.*, 2020). This proposes that the variables contributing to yield are relevant in determining the overall variation identified in the dataset. The third principal component accounted for 12.27% of the total variation, associated positively with leaf area (0.505), leaf width (0.443) and leaf length (0.411), and negatively associated with internodal length (-0.652) and petiole length (-0.627). Number of immature berries spike$^{-1}$ (0.566) and wax content (0.555) were shown to be associated positively in PC4 which explained 8.26% variability.

**Table 1.** Eigen values and percentile variance of selected black pepper genotypes

| | Principal Component | | | | |
|---|---|---|---|---|---|
| | PC 1 | PC 2 | PC 3 | PC 4 | PC 5 |
| Eigen value | 5.24 | 2.53 | 2.08 | 1.4 | 1.28 |
| % Variance | 30.87 | 14.9 | 12.27 | 8.26 | 7.56 |
| Variable | Eigen vectors | | | | |
| Leaf length (cm) | -0.587 | 0.022 | 0.411 | 0.232 | 0.379 |
| Leaf width (cm) | -0.705 | -0.095 | 0.443 | 0.026 | 0.349 |
| Leaf area ($cm^2$) | -0.742 | -0.04 | 0.505 | 0.119 | 0.403 |
| Petiole length (cm) | -0.414 | 0.255 | -0.627 | 0.144 | 0.154 |
| Internodal length (cm) | -0.255 | 0.355 | -0.652 | 0.078 | 0.348 |
| No. of stomata | -0.53 | 0.227 | 0.31 | -0.284 | -0.358 |
| Wax content ($\mu g/cm^2$) | 0.506 | -0.065 | -0.17 | 0.555 | -0.036 |
| Number of spikes $plant^{-1}$ | 0.592 | 0.279 | 0.14 | 0.219 | -0.043 |
| Test weight (gm) | 0.527 | 0.73 | 0.332 | -0.039 | 0.036 |
| Spike length (cm) | 0.753 | 0.171 | 0.356 | 0.202 | 0.077 |
| Peduncle length (cm) | 0.468 | 0.239 | 0.074 | -0.51 | 0.065 |
| Berry size (mm) | 0.486 | -0.648 | 0.084 | -0.429 | 0.07 |
| Number of matured berries $spike^{-1}$ | 0.523 | 0.752 | 0.224 | -0.092 | 0.113 |
| Number of immature berries $spike^{-1}$ | 0.061 | -0.276 | 0.35 | 0.566 | -0.476 |
| 100 berry fresh weight (g) | 0.537 | -0.654 | 0.104 | -0.145 | 0.203 |
| 10 spiked berries weight (g) | 0.693 | -0.082 | 0.138 | 0.101 | 0.439 |
| 10 rachis weight (g) | 0.623 | -0.289 | -0.226 | 0.158 | 0.305 |

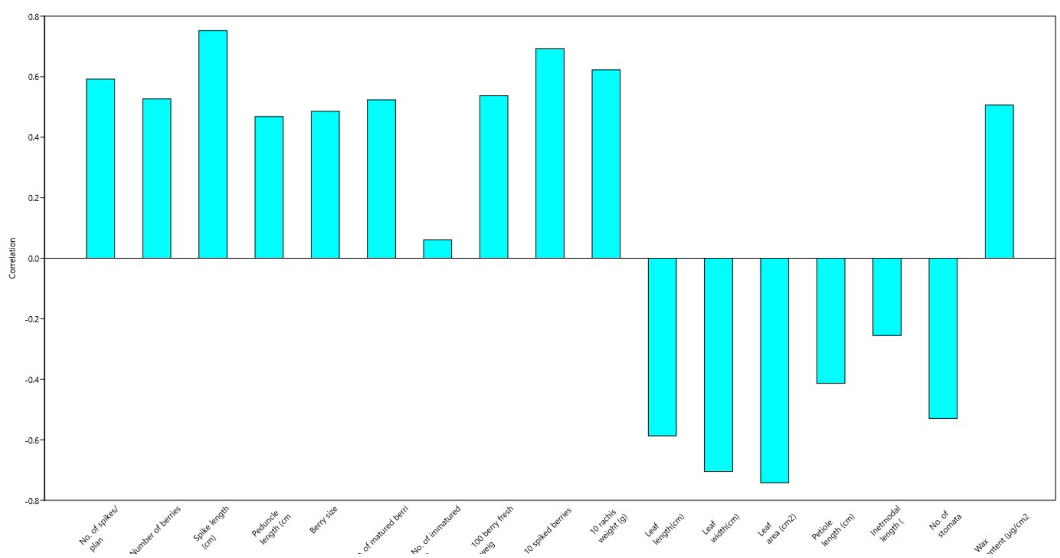


**Fig. 2.** Loading plot of first principal component with variables

Scatterplot helped to visualize the genotypes grouping based on similarities and differences according to the influence of traits. Except the number of immature berries spike$^{-1}$ in yield attributing characters, number of spikes plant$^{-1}$, test weight, spike length, peduncle length, berry size, number of matured berries spike$^{-1}$, 100 berry fresh weight, 10 spiked berries weight and 10 rachis weight including the wax content mainly attributed to the first axis as positive levels (Fig. 3). Shivakumar *et al.* (2022) observed that berry weight and dry seed weight were highly significant among various black pepper genotypes for spike and berry traits, displaying a strong positive correlation through principal component analysis.

The traits that made the most significant contribution to the first principal component were genotypes having drought tolerant characteristics *viz.* accession 971, closely followed by accessions 4132, 7211, 6720, 1343, and 1086. Whereas negative contribution was made by the traits, which highly correlated with the accessions (5083, 4064, 1491, 8060 and 1093) with susceptible characteristics. Among all the genotypes considered, accession 4132 displayed the positive value in first component and most negative value in the second principal component, indicating that it possessed the most tolerant characteristics. Malek *et al*. (2021) observed that the genotypes could be distinguished based on the key contributing features using the biplot described by the first two PCs.

Hence, it can be concluded that morphological characteristics and stomatal density, which were distributed across the second and third quadrants, exhibited both significant and non-significant negative associations with the traits that contribute to the yield and wax content of black pepper genotypes compared to those in the first and fourth quadrants. It is assumed that drought tolerant black pepper genotypes will have reduced leaf area, reduced internodal and petiole lengths, lower stomatal density and higher wax content. Genotypes displaying traits that make them vulnerable to drought were placed in the second and third quadrants.

An effective visual depiction of the relationship between variables in a dataset can be obtained by using a Pearson correlation analysis. Pearson correlation analysis revealed a significant relationship among observed morphological traits. According to the present study, there was a strongest positive correlation ($P<0.001$) between the leaf area and leaf width ($r= 0.93$) which is in consistent with the findings reported by Preethi *et al*. (2018), followed by leaf length ($r= 0.80$) as well as between internodal length and petiole length ($r= 0.60$) ($P<0.01$). Leaf area showed a consistent relationship with the leaf width (Jayarathna *et al*., 2016).

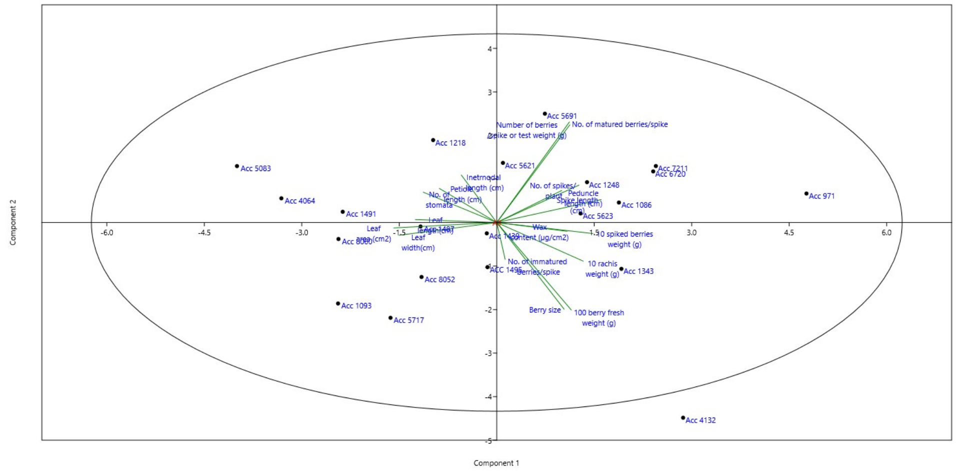


**Fig. 3.** Black pepper genotypes distribution across the first two primary components

Larger leaf area was not positively correlated to the improved cultivar production, although the flower intensity spike$^{-1}$, inflorescence size and berry spike have positive correlation with fruit development (Chen *et al*., 2018). Water deficiency lead to reduction in both leaf size and quantity in black pepper plants (Rasanjali *et al*., 2019; Teles *et al*., 2023). Similar findings have been reported in tomato (Heuvelink *et al*., 2005) and pepper crops, indicating a reduction in leaf area due to water shortage (Cemek *et al*., 2020; Koch *et al*., 2019). Hence, a reduction in leaf area can be regarded as a favourable characteristic for drought tolerance as reduced leaf area reduces the water loss through transpiration, supported by the present study.

A homogeneous association was shown between leaf length and width (r= 0.54). While non- significant positive correlation (P≥0.05) was showed by stomata with leaf length (0.20), leaf width (0.33) and leaf area (0.36). Wax content exhibited high negative correlation with stomata number (-0.49) (P<0.05), followed by leaf width (-0.42), leaf area (-0.39) and leaf length (-0.18). Black pepper varieties with broader and larger leaf bases typically have more stomata (Paulus & Sim, 2011) as shown by the leaf length to width ratio. Elevated stomatal density could be associated with the leaf area expansion and cell division. Enhancing regulatory competence of stomatal traits through an approach of selective breeding strategies might help plants to respond more efficiently to environmental constraints like drought (Xu & Zhou, 2008). While experiencing drought and excessive radiation, a decrease in transpiration rate and an increase in leaf wax load have a positive impact on the yield index (Sanchez *et al*., 2001). The wax content demonstrated the non- significant positive relationship with the traits that contribute to yield. The positive correlation between leaf wax content and yield-attributing traits provides valuable insights into a plant's ability to conserve water content in the best yield-producing genotypes especially under water deficit. This information may be used in breeding strategies to enhance production under water limited environment.

Among yield attributing traits, highly significant and positive correlation (P<0.001) was found between berry size and 100 berry fresh weight as well as test weight and number of matured berries/ spike (r= 0.83). The fresh yield was found to have a positive correlation with the number of berries per spike (Sainamole *et al*., 2002; Bermawie *et al.*, 2019). The association between berry and test weight could be an indication of overall yield, emphasizing their importance in determining the yield.

Significant positive correlations (P<0.01) were identified between test weight and spike length (r= 0.61), 10 rachis weight and spike length (r= 0.59) and number of matured berries/spike and spike length (r= 0.57). The number of berries spike$^{-1}$ (Ibrahim *et al*., 1985) as well as spike length (Krishnamurthy *et al*., 2010) can be affected by both genetic and environmental factors. The quantity of berries shows greater responsiveness to changes in environmental conditions compared to the length of the spike, further supporting and extending the understanding provided by Ibrahim *et al*. (1987). The current study focused on the interaction of genetic and environmental variables, especially influencing berry size, test weight, spike length, and the number of matured berries per spike. The number of berries in a spike is directly associated with the length of the spike, a correlation observed consistently across different states such as Kerala (Maheswarappa *et al*., 2012; Sujatha and Namboothiri, 1995; Reshma *et al*., 2022), Karnataka (Tripathi *et al*., 2018), and Assam (Deka *et al*., 2016; Nath *et al*., 2021). Rachis weight, spiked berries weight, 100 berry fresh weight, spike length, number of spikes per plant and peduncle length were non- significantly and positively correlated with each other. Number of berries per spike directly and positively contributes to the pepper yield.

Shivakumar *et al*. (2022) reported that Panniyur-1, Agali, and Narayakodi genotypes exhibited high values on PC1, and these genotypes displayed higher values for yield-attributing traits (berry weight, dry seed weight, and fresh pericarp weight), indicating a strong positive correlation as observed in the present study. These traits hold relevance in both principal compont analysis and clustering of genotypes.

Results of the morpho-physiological and yield contributing traits based dendrogram (Fig. 4) in the present study displayed genotypes with most preferable traits for the drought tolerance in clusters 2 and 3, while genotypes with susceptible characteristics were grouped in cluster 1. The accessions 7211, 971 (cluster 2), 4132, 1495, and 1343 (cluster 3) had traits suited for drought tolerance. The present study is in conformity with previous findings in wheat genotypes that were categorized based on morphological traits by the resultant dendrogram into high homogeneity clusters within the clusters (Pasandi *et al*., 2016). The attributes responsible for wheat yield like spikes plant $^{-1}$, number of grains spike $^{-1}$, grain weight spike $^{-1}$, grain yield plant$^{-1}$, and spike density made substantial contributions to the first principal component and were grouped together in the same cluster (Fouad, 2020). The variation among black pepper genotypes for yield contributing traits as per the PCA and further classification of genotypes by dendrogram in the present study might reflect their genotypic variations as well as the environmental influences.

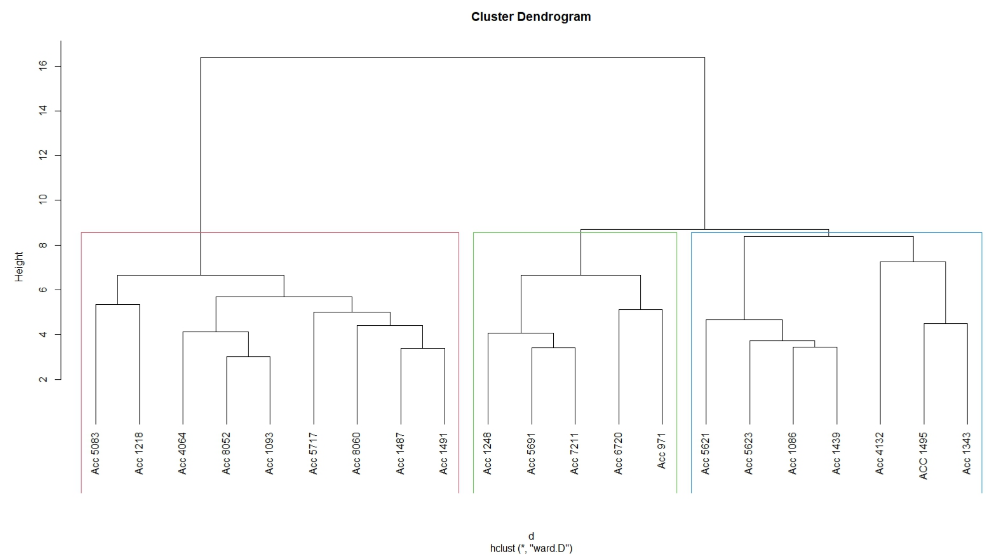


**Fig. 4.** Clustering of black pepper genotypes

In conclusion, the current study confirmed significant genetic variability among the selected black pepper genotypes which is useful to select genotypes with desirable drought-tolerant characteristics. The identification of factors that contribute significantly to PC1 can be helpful in identifying black pepper genotypes with drought-tolerant characteristics with sustainable yield. The genotypes 4132, 7211, 1343, 1495 and 971 were identified as having better drought-resilient traits. This knowledge can also be used to develop targeted breeding and cultivation strategies aimed at selecting genotypes with specific drought-tolerant traits. Ultimately, this can lead to the development of black pepper variety tolerant to drought, resulting in improved yields and sustainable black pepper production.

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