Journal of Underutilized Legumes
Vol. 6 (1): 1 – 16 (March, 2024)

Society for Underutilized Legumes
https://sulegumes.org/
e-ISSN: 2705-3776

Resilience in Hyacinth Bean (*Lablab purpureus* (L.) Sweet): A Combined Approach of Relative Water Content and Gene Expression Analysis for Drought Tolerance.

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Received January 6, 2024
Accepted for publication March 9, 2024
Published March 12, 2024

Abstract

To cope with water deficit, plants have evolved diverse drought tolerance mechanisms that involves physiological, biochemical and gene regulatory networks for their effective survival. This research investigated the relative water contents (RWC) and semi-reverse quantitative expression of plasma membrane intrinsic proteins (PIPs) drought-tolerance genes in Hyacinth bean (HB). Five accessions were randomly selected based on their seed morphology and were grown in planting buckets filled with topsoil in a randomized complete block design. After two weeks of seedling growth under normal conditions, drought stress was induced without water for 21 days in a greenhouse. In the same experiment, well-watered potted HB seedlings served as controls. Determination of the RWC followed an established protocol. RNA was isolated from leaf sample of the accessions and mRNA expression levels of the PIP gene was determined using real-time quantitative PCR (RT-qPCR) with GAPDH (glyceraldehyde – 3- phosphate dehydrogenase) gene as control. The results showed that the RWC of drought-treated accessions ranged from 26.17% (TLn-6) to 48.78% (TLn-2), while that of control varied from 49.61% (TLn-2) to 79.07% (TLn-70). The mean cycle quantification for the PIP amplification in the drought-treated samples ranged from 31.33 ± 0.079 (TLn-2) to 32.02 ± 0.364 (TLn-7) which were found to be higher than the control (28.63 ± 0.00).The expression level of drought-treated accessions was higher than that of the control accessions. The expression of PIP in both drought-induced and well hydrated accessions suggest the presence of drought regulatory genes in HB, thus making the crop a suitable candidate for climate-smart agriculture in the tropics.

Keywords: Drought-tolerance; gene expression; Hyacinth bean; water stress

Introduction

The Fabaceous Hyacinth bean (HB) (*Lablab purpureus* (L.) Sweet) is a highly proteineous diploid legume with a 2n = 22 chromosomes configuration [1,2]. The plant is widely distributed in Africa and Asia while it is considered an introduced species in Queensland Australia, Italy, Central and Southern African countries [3]. HB is adapted to unfavourable environments and climatic conditions (4). It is a multipurpose crop extensively grown for its green pods, proteinaceous seeds and leaves [5,6]. Lablab with its abundant micro and macronutrients is touted by experts as a candidate crop to combat malnutrition and food insecurity, especially in rural communities of Africa [7,8]. Its nutritional content, digestibility, antioxidant, pharmacological, physicochemical and phytochemical properties have been well characterized [3,6–9]. The cultivation of Lablab serves as valuable income source to peasant farmers, forage for ruminant animals, and green manure to enhance soil fertility [6,12]. Recent reports have indicate that edible Lablab contains carbohydrate-binding proteins capable of impeding viral infections such as influenza and SARS-COV-2 [9,13,14].

Hyacinth bean (HB) is one of the neglected and underutilized legumes (NULs) which exhibit good agronomic traits for higher yield via its papilionaceous flowers and long pods [15,16]. Asides the agronomical values of NULs, incorporating HB as a food crop with multifunctional uses will help in sustainable agriculture and food security. HB is adaptable to harsh conditions such as drought and salinity stress by reducing the number and weight of nodules, morpho-physiological adjustment at seedling stage and elevated oxidative stress markers [16–18]. Physiological factors such as reduction in relative water content (RWC) is an adaptive mechanism effective in alleviating drought stress in legumes [19,20]. Reduction in RWC is among the key physiological parameters for choosing high yielding accessions as this helps in cell turgor pressure maintenance under drought [21]. Hence, RWC determination is a screening tool for drought tolerance in crop production. The intensity and frequency of drought have increased due to climate change, understanding how crops can cope with changing climatic conditions will help address higher crop productivity in terms of food security, crop breeding and genetic improvement, resource conservation, and optimizing water use. As different regions in the world faces varying degrees of drought, understanding how crops can withstand drought conditions will ensure a stable global food supply.

Scientific knowledge on HB genetic diversity, agronomic practices, breeding, and utilization is low compared to notable legumes [22]. The plant is considered a lost crop and under-used by reason of little research and development interest, yet it has the potential of becoming an important resource for the agricultural systems in the tropics to improve both human food and animal feedstock [23]. Lablab could be exploited to achieve food security, diversify diets, increase smallholder income, boost nutrition and health, improve ecosystem services and 'climate-smart' agriculture [6,24]. Despite its broad potential, cultivation and production are steadily declining due to eroding cultural practices, low demand, unawareness of its nutritional composition, lack of domesticated varieties and poor management practices [25,26].

The characterisation of genetic variation in plant germplasm resources is fundamental to their conservation and breeding. Genetic variation arises due to differences in genetic composition (DNA sequences and gene forms), and forms the basis for variations in morphological, physiological, and biochemical attributes. Gene expression can be influenced by environmental conditions. The principal contributors to genetic variation are mutation and meiotic recombination alongside other factors such as selection (natural and artificial), genetic drift and gene flow [27]. The genetic diversity of Lablab accounts for its extreme phenological plasticity, which is essential in developing mechanisms to cope with drought [15,24]. Presently, Lablab is among the 101 candidate crops selected by the African Orphan Crops Consortium (AOCC) for high throughput sequencing to generate sequence data valuable to improvement of the species [23,28]. Agro-morphological markers and different DNA marker techniques including AFLP, RAPD, Diversity Array Technology (DArT), EST-SSR, ISSR and SSR have been deployed to gain insight into the

genetic diversity of Hyacinth bean across different regions [23,26,29–31], studies that combine biochemical profiling and gene expression for drought tolerance are limited [8,23].

Plants undergo several biotic and abiotic stress factors under natural and agricultural conditions [32–34]. The uptake and transport regulation of water across tissues and cellular membranes is crucial for plant growth and development [35]. It is now known that water stress can reduce crop biomass production and overall productivity by about 50% (36–38). To cope with water deficit, plants have evolved diverse drought tolerance mechanisms that involves physiological, biochemical and gene regulatory networks for their effective survival [38]. Drought tolerance involves maintaining turgor pressure by adjusting osmosis, increasing flexibility, and decreasing the size of the cell by protoplasmic resistance. Some xeromorphic features, such as hairy leaves, and the formation of trichomes on both sides of leaves, can be seen in drought-tolerant plants during water-stress conditions. While transpiration enhances light reflection and reduces water loss, hairiness lowers temperature [39]. Aquaporins (AQPs) are members of the large group of the Major Intrinsic Proteins (MIP) known for role they perform in water and solute transport in almost all living organisms [40,41]. In angiosperms, the AQPs are categorized into five subfamilies that include small basic intrinsic proteins (SIP), NOD26-like intrinsic proteins (NIP), plasma membrane intrinsic proteins (PIP), tonoplast intrinsic proteins (TIP) and X intrinsic proteins (XIP) [35]. Plant AQP gene expression is differentially modulated in different in tissues and is also modified under different environmental and physiological stresses [35]. Lablab is known for its drought-tolerant ability, credited to its high phenological and morphological plasticity [4]. Thus, it is recommended for drought-prone locations, as it can survive in water scarcity while maintaining good yield compared to other grain legumes. This study investigated the relative water contents (RWC) and expression pattern of the drought-tolerance genes encoding plasma membrane intrinsic proteins (PIPs) in Hyacinth bean accessions under water stress.

Materials and Methods

Seed acquisition and physical characterization

Twenty accessions of Hyacinth bean (HB) were obtained from the Genetic Resources Centre of the International Institute of Tropical Agriculture (IITA) Ibadan, Nigeria via the Standard Material Transfer Agreement (SMTA). The phenotypic images of the seeds are shown in Plate 1. Five different accessions based on seed coat colour were selected for this study.

Plate 1*:* Phenotypic Image of the twenty Lablab Accessions

Planting, growth, and collection of leaf samples

Seeds of five different accessions of HB based on seed colour morphology (TLn-2, TLn-6, TLn-7, TLn-51, and TLn-70) were selected from Plate 1 for relative water contents and molecular characterization. The seeds were scarified using a razor blade to aid seed germination and were grown in pots filled with topsoil in a randomized complete block design in three replicates. The seedlings were subjected to water stress (lack of water) for 21 days in a green-house. In the same experiment, well-watered potted HB seedlings (irrigated using a 75 cl of water at interval of 12 hours) served as controls (Plate 2 and 3). Data such as leaf length, width and plant height were collected before harvesting leaf samples. Leaves from three weeks old seedlings of both the control and experiment were harvested in well-labelled Ziploc bags for gene expression analysis.

Plate 2: The Control accessions; TLn-2, TLn-6, TLn-7, TLn-51, TLn-70 21 days after planting (irrigated with 75 cl of water at interval of 24 hours).

Preservation of leaf tissues

Leaf samples of the accessions were harvested at three weeks old (21 days). About 0.1 to 1g of leaf samples were inserted in labelled Eppendorf tubes. In order to preserve the leaf tissues, 600 µl of RNA/DNA shield (ZYMO RESEARCH) was added to the leaves until the leaves were fully immersed. The samples were stored in -80℃ freezer until samples were shipped to Inqaba West Africa (Ibadan) laboratory for analysis.

Plate 3: Aerial view of drought-induced accessions for 21 days (no water at all – water deficit); TLn-2, TLn-6, TLn-7, TLn-51, TLn-70.

Determination of Relative Water Content (RWC)

The RWC was investigated in the leaves of both the control (watered samples) and experiment (water stressed (dehydrated) – without water for 21 days) setups of the accessions. Three leaves were randomly collected from three replicates of each accession and preserved in Ziploc bags while transported to the laboratory. Fresh weight of the leaves was measured and recorded. The leaves were immersed in water for 5 hours to achieve full turgidity(rehydrated by floating on de-ionized water in a close petri dish). Afterwards, the leaves were dabbed remove surface moisture and weighed. The recorded weight served as the saturated weight. Subsequently, the leaves were laid on a well labelled aluminium foil in an oven (Genlab MINO/50, Cheshire, UK) to dry at 105℃ for 15 min. The oven temperature was later adjusted to 65℃ and the leaves were left to dry for 48 hours. After drying, the leaves were weighed to obtain their dry weights. To evaluate the RWC percentage in for each obtained per accession, the following formula was applied and as described by Pieczynski et al. [33] and Soltys-Kalina et al. [34].

$$RWC\% = \frac{FRESH\ WEIGHT - DRY\ WEIGHT}{SATURATED\ WEIGHT - DRY\ WEIGHT} \times 100\%$$

RNA Isolation and quantification

Ribonucleic acid (RNA) was isolated using the Quick-RNA Miniprep Plus Kit (Zymo Research, Orange, California) following the Manufacturer's procedure. The quality and quantity of the extracted RNA were measured using a nanodrop (Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer, Massachusetts,

America). The spectrophotometer was blanked using 1 uL of RNA Elution Buffer. Afterwards 1 uL of the RNA was placed on the pedestal and measured.

Real time – qualitative PCR (RT-qPCR)

The presence of Hyacinth Bean AQP was detected using RT-qPCR. Primers targeting the AQPs of PIPs (Table 1) were synthesized by Inqaba West Africa Ltd, South Africa. The PCR for the samples was performed in duplicates in the BioRad CFX 96 touch system using the Luna® Universal One-Step RT-qPCR Kit (New England Biolabs) following manufacturer's instructions. The total reaction volume of 20 µL contained 1 µL of RNA template, 0.8µL each of 10µM forward and reverse primers, 10µL of Luna Universal One-Step Reaction Mix (2X), 1.0µl of Luna WarmStart® RT Enzyme Mix and 6.6 µL of Nuclease free water. The tubes were thereafter spun down to collect the liquid and placed in a real-time thermocycler. The reaction was done as follows: reverse transcription at 55 Degree Celsius (℃) for 10 mins, initial denaturation at 95℃ for 1 mins, both of which runs for cycle 1, denaturation at 95 Degree Celsius for 10 secs and extension at 60℃ for 30 seconds and both runs for 40 – 45 cycles while the melt curve temperature was at 60 – 95℃ for various minutes and 1 cycle. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a standard control. The PCR products were loaded into 1% agarose gel to ensure a single target was amplified (Plate 4). Gene expression data were analysed by the CFX Manager Software Version 3.1 using the $2^{-\Delta\Delta CT}$ method.

Table 1: Sequences of primers used for this study. Primer design

Name	Oligo sequence	Number of bases	Tm °C	Amplicon size
PIP2-1F	CTGTTTTGGCACCACTACCC	20	59	103
PIP2-1R	GATCCGAAACTTCTTGCCGG	20	59	

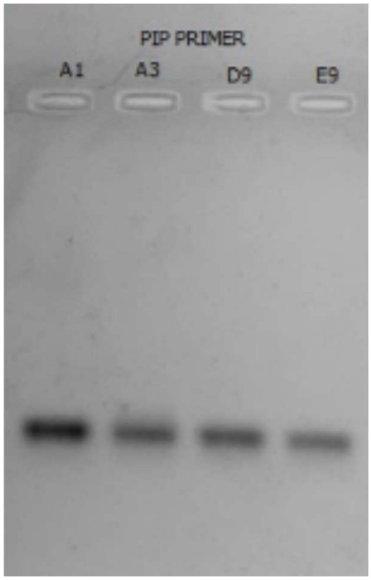

Plate 4: Gel image of the RNA extracted from the plant samples(A1 = TLn-2, A3 = TLn-6, D9 = TLn-7 and E9 = TLn-70).

Results

Relative Water Content of leaves

The results showed that the RWC of drought-treated accessions ranged from 26.17% (TLn-6) to 48.78% (TLn-2), while that of control varied from 49.61% (TLn-2) to 79.07% (TLn-70) (Figure 1).

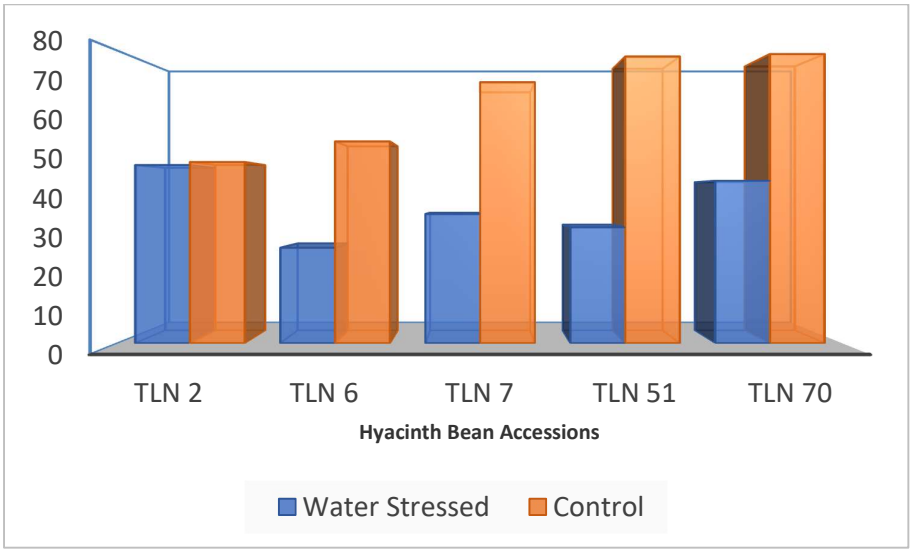

Figure 1: Graphical representation of relative water contents for the accessions at 21 days old.

PCR efficiency of Target genes

The amplification showed 100% efficiency for the GAPDH and PIP genes which is an indication that the enzyme worked maximally as demonstrated by the normal amplification curve presented on Figure 2 and Table 2.

PIP gene expression quantification

The mRNA expression levels of the PIP genes were determined using real-time RT-qPCR with GAPDH (glyceraldehyde – 3- phosphate dehydrogenase) gene as control. To carry out the amplification, the treatment and control samples were comprised of 4 replicates. The PIP subfamily drought tolerance gene was successfully expressed in both watered and water-stressed samples. The mean cycle quantification for the PIP amplification in the drought-treated samples ranged from 31.33 ± 0.079 (TLn-2) to 32.02 ± 0.364 (TLn-7) which were found to be higher than the control (28.63 ± 0.00) (Table 3). The Table described the expression of PIP in both drought-induced and control accessions of HB. The cycle of amplification of PIP and GAPDH is shown in Figure 2. The bar graphs of normalized gene expression levels of PIP are depicted in Figure 3.

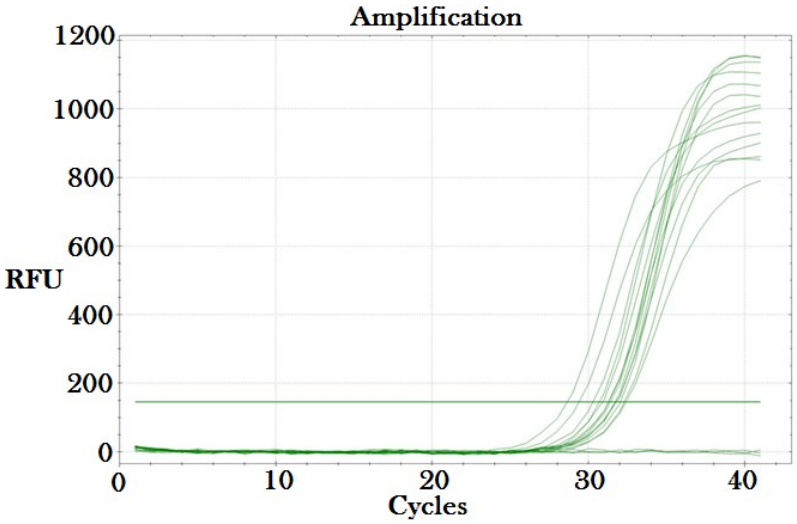

Figure 2*:* Cycle Quantification results of the amplification of PIP and GAPDH

Table2: Efficiency of PCR of target genes.

Name	Full Name	Reference	Auto Efficiency	Efficiency (%)
GAPDH	GAPDH	TRUE	Yes	100.00
PIP	PIP	FALSE	Yes	100.00

GAPDH - Glyceraldehyde – 3- phosphate dehydrogenase gene, PIP - plasma membrane intrinsic protein

Table 3: PIP expression quantification among the accessions.

Target	Sample	Expression	Expression SEM	Corrected Expression SEM	Mean Cq	Cq SEM
GAPDH	5E	N/A	N/A	N/A	31.82	0.55906
PIP	1E	2.29343	0.71696	0.71696	31.33	0.05551
PIP	3E	4.02171	1.42482	1.42482	32.02	0.25742
PIP	5C	0.78437	0	0	31.68	0

Where 5E = TLn – 70 treated, 1E = TLn – 2 treated, 3E = TLn – 7 treated, 5C = TLn – 70 Control. GAPDH - Glyceraldehyde – 3- phosphate dehydrogenase gene, PIP - plasma membrane intrinsic protein, N/A = Not Available.

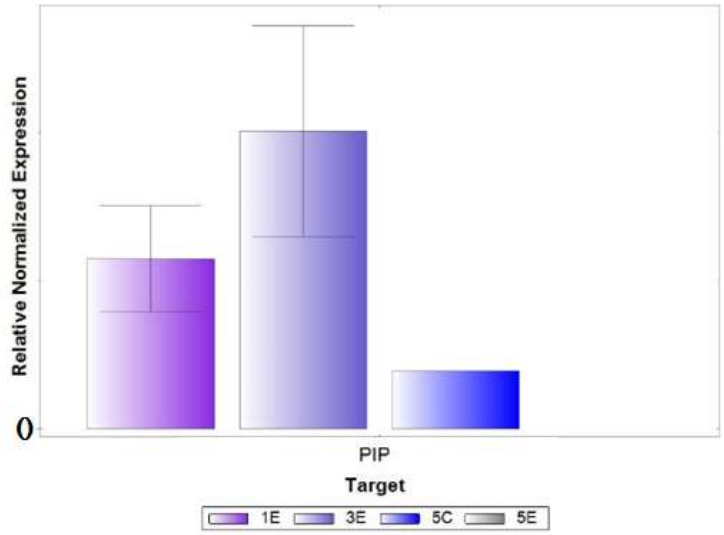

Chart Error: ± 1.0 SEMs

1E: TLn-2 treated 3E: TLn-7 treated 5C: TLn 70 control 5E:TLn-70 treated

Figure 3: Bar graphs of Normalized gene expression levels of PIP in the accessions.

Discussion

Relative water content (RWC) is one of the most accurate methods in determining the status of the water content as it pertains to cellular hydration [43,44]. RWC measures the current water content within a sample relative to its maximal water retention capacity at full turgidity. Water, among others, is an essential component in plants' life required for growth, development, metabolism, and other physiological processes. Drought is water-related stress, often used to describe a prolonged period without rainfall or water supply [45]. Globally, drought stress inhibits crop production by reducing soil water potential, induce cell dehydration, lowers the uptake of nutrients for effective stem and leaf growth [46]. In this study, HB accessions were hydrated with water and also dehydrated (denied water) for a period of three weeks to detect expression of plasma membrane intrinsic proteins (PIPs) known as drought-tolerance genes. Varied RWC values were recorded for both hydrated and water-stressed (dehydrated) samples while PIPs were expressed in both samples.

Our results indicate that the RWC values for the control samples (hydrated) were higher than the values recorded for the water-stressed samples (dehydrated). RWC of hydrated accessions (control) varied from 49.61% in TLn-2 to 79.07% in TLn-70 whereas water-stressed accessions ranged from 26.17% in TLn-6 to 48.78%. Generally, in well-hydrated plant samples, leaf RWC can range from 98% to 99% and in water-stressed samples, it can range between 30% to 40%, however, depending on the crop [47]. Khan et al. [48] reported a low RWC in water-stressed and high RWC in watered (hydrated) accessions of Faba bean (*Vicia faba* L.), a closely related legume species to Hyacinth bean. According to their findings, a low RWC in drought stressed plants could be attributed to decreased activity of cells, stomatal closure, slow root growth and possible accumulation of abscisic acid within guard cells. Similarly, Guretzki and Paperbrock [49] reported that RWC reduces as a result of water scarcity in Hyacinth bean which correspond to the findings of this study. While the RWC values for the treated samples agrees with the general range of approximately 30% to 40% as highlighted above, the RWC values for the control show great disparity from the normal range for well-watered plants of 98 to 99%. This disparity could be attributed to the frequency

of irrigation which might have increased water contents of the HB accessions and as reported in common beans [50]. Among the control accessions, TLn – 70 exhibited a higher value of RWC (79.07%) which could be linked to the high metabolic rates among the well-watered (control) samples. In contrast, water-stressed samples recorded a lower RWC values which ranged from 26.17% in TLn – 6 to 48.79% in TLn – 2.

Gene expression is an important process regulated by the central dogma of life. It enables cellular response to environmental change and protein formation. Gene expression analysis is crucial in understanding the structure-to-function relations of protein and their regulation. The PIP subfamily of the AQPs was successfully amplified and expressed in both hydrated and dehydrated samples. PIPs are known to be involved in abiotic stress responses and are naturally highly controlled at the transcriptional level, responding to various range of developmental and stress related signals [42,52]. The expression levels in the samples treated with water-deficit stress (dehydrated) were higher than the control. The expression level of treated (dehydrated) TLn-7 (3E) (4.02171) was almost four times higher than that of the control sample (hydrated) 5C (0.78437) and the expression level of TLn-2 (1E) 2.29343) was twice as much as the expression of the control sample (0.78437). A higher expression levels of PIP in drought-treated legumes such as common bean and Faba bean has been reported which conforms to the present findings on Hyacinth beans [21,43,48,49]. There is a link between a low RWC recorded in dehydrated samples (water-stressed) and a higher expression levels of PIPs in the same samples. D'Souza et al. [52] investigated responses of HB to drought stress and reported a link between the PIP water channels and the relative water content of the plant under drought-induced and well-watered conditions. A reverse transcriptase-PCR examination of the gene expression profile of the leaf tissues indicated that PIP genes were expressed in both hydrated and dehydrated plants similar to the findings of this study. However, the PIP genes were more abundant in the tissues of the dehydrated plants than in the hydrated plants (control). A low RWC value and expression of drought tolerance gene proline in cowpea proves as a key indicator for water deficits [53]. In a related study, drought sensitive *Pisum sativum* variety was shown to express PsDREB2A (a family of DREB gene) with a decrease in leaf relative water content under drought [54]. In another study, aquaporin (AQP) proteins in two cultivars of *Phaseolus vulgaris* were down-regulated under drought stress with higher leaf RWC in drought-tolerance cultivar than the drought sensitive cultivar [55].

The combined approaches of physiological and expression of drought tolerance gene in HB is vital to utilization of marker-assisted breeding programme allowing for the selection of accessions with desirable drought resistant traits. The understanding gained from this integrated approach will guide researchers in engineering HB germplasm with specific adaptations, enhancing their ability to withstand drought stress, accelerate its improvement and also reveals how the crop will respond to drought in specific environmental conditions. Thus, enhancing drought tolerance in HB has potential application in plant breeding to increase yield and food security, mitigate climate change, and sustainable agriculture.

AQPs of the subfamily PIP were expressed in both drought-induced and well hydrated samples which also relate to the relative water contents of the accessions. As previously pointed out, a low RWC value and expression of drought tolerance genes attest to the adaptive features of HB to drought prone areas. The presence of drought regulatory genes makes it a climate-smart crop that can rapidly adapt to climatic changes. The expression of PIP in both drought-induced and well hydrated accessions suggest the presence of drought regulatory genes in HB, thus making the crop a suitable candidate for climate-smart agriculture in the tropics. Nevertheless, it is expedient for stakeholders including farmers, breeders, scientists, and other researchers working on HB to harness its potentials for an enhanced drought tolerance to achieve food security, sustainable agriculture, and environment in the near future. Finally, this paper call for action for a continued research and innovation using emerging biotechnological means such as gene editing, genomic, and proteomic tools to advance the physiological and genetic potentials of HB for sustainable utilization in Africa and beyond.

Author's Contribution

Jacob O. Popoola: Conceptualization, Supervision, experiment planning, Data analysis and interpretation, writing original draft, Reviewing, and Editing. Ifemazi Chioma Faith: Carried out the experiments and Data analysis. **Abiodun S. Oyelakin, Oluwadurotimi S. Aworunse,** Omena Bernard .Ojuederie, James O. Agbolade**:** Writing-Reviewing and Editing. **Idowu Arinola Obisesan:** Literature review and editing, **Ramar Krishnamurthy**: Technical Expertise, Guidance, Reviewing and Editing.

Acknowledgements

Genetic Resources Centre, International Institute of Tropical Agriculture (IITA), Ibadan, Oyo State, Nigeria for providing the seeds for this work.

Declaration of Competing Interest

The authors declare that there is no competing interest that could have appeared to influence the work reported in this paper.

Funding

This research work did not receive funding.

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