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DAB assay: A simple, rapid and reliable protocol for screening for water stress - case study of common bean and cowpea

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ABSTRACT

H_2O_2, induced due to biotic and abiotic stresses, is a strong oxidant and can initiate localized oxidative damage in leaf cells leading to disruption of metabolic function and loss of cellular integrity resulting in senescence. Staining with DAB (3,3-Diamainobenzidine), which is an efficient marker of H_2O_2 accumulation *in vivo*, can be reliably used for delineating stress response in crop plants. In the present paper we validated the utility of DAB assay for stress screening by evaluating 50 genotypes of common bean and 40 genotypes of cowpea under irrigated and drought conditions. In both the crops, the assay clearly differentiated the lines on the basis of darker staining of leaves under drought. The lines showing greater per cent reduction in yield parameters showed greater staining in DAB assay underlining the reliability of using this assay as a supplement to phenotyping protocols for characterizing large germplasm sets. The assay is cost effective as compared to spectophotometric methods of assessing oxidative damage, less time consuming and can be replicated across crops. The detection of cellular levels of H_2O_2 using DAB staining method and our results reveal a clear difference in degree of staining achieved in the stressed plant. It can be safely concluded that DAB staining can be an efficient biochemical tool for screening for water stress in legume crops.

Key words: DAB assay, Legumes, Oxidative damage, Water stress

INTRODUCTION

Water stress induces the accumulation of reactive oxygen species (ROS) in plants (Vickers *et al.,* 2009) mainly produced in chloroplasts and peroxisomes (Kim *et al.,* 2008). ROS's play an important role as signaling molecules that initiate stress response in plants. Environmental stresses are known to induce H_2O_2 and other toxic oxygen species production in cellular compartments, resulting in acceleration of leaf senescence through lipid peroxidation and other oxidative damage (Upadhyaya *et al.* 2007). Being a strong oxidant H_2O_2 can initiate localized oxidative damage in leaf cells leading to disruption of metabolic function and loss of cellular integrity. In plant cells, H_2O_2 is produced by univalent reduction of O^{2-}. Excessive production of H_2O_2 triggers oxidative stress due to its ability to inactivate antioxidant enzymes by oxidizing their thiol groups (Gill and Tuteja, 2010). Out of the various forms of ROS, H_2O_2 plays a key role in plant signaling and adaptation to abiotic and biotic stresses (Foyer and Noctor, 2012). Increased production of H_2O_2 is commonly observed feature of plant stress response signature (Formentin et al, 2018). Crop varieties reported to be less tolerant have been reported to accumulate higher amounts of H_2O_2 with resultant increase in the lipid peroxidation (Zlatev *et al.,* 2006, Sofi et al, 2017, Gull et al, 2019).

DAB (3,3'-Diaminobenzidine) is an organic compound with the formula $(C_6H_3(NH_2)_2)_2$. It is a derivative of benzidine, and a precursor of polybenzimidazole. It is a water-soluble tetra-hydrochloride of poly-benzimidazole, and is routinely used in immuno-histochemical staining of nucleic acids and proteins (Jovicic et al, 2015). The molecular weight of DAB is 214.27 g/mol and its molecular formula is $C_{12}H_{14}N_4$ or $(NH_2)_2C_6H_3C_6H_3(NH_2)_2$

DAB assay is used in normal pH but is highly sensitive to light. It is also used in *in situ* hybridization (ISH) and sometimes in dot blots and in western blotting. In DAB staining, DAB is oxidized by hydrogen peroxide in a reaction typically catalyzed by horseradish peroxidase (HRP). The oxidized DAB forms a brown precipitate, at the location of the HRP, which can be visualized using light microscopy. The DAB precipitate is insoluble in water, alcohol, and other organic solvents such

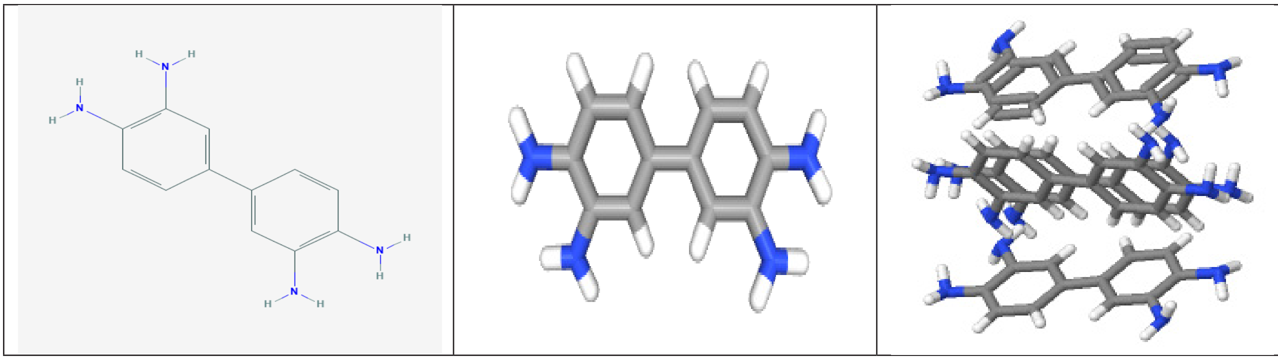


Figure 1: (https://pubchem.ncbi.nlm.nih.gov/compound/3_3_-Diaminobenzidine)

as xylene and isopropanol. This allows for great flexibility in the subsequent treatment of the tissue section, such as in the choice of counter stain and mounting medium. DAB staining is also heat-resistant, so it can be used in double labeling IHC/ ISH experiments, and is extremely stable – in fact stained slides are often stable for many years (Jovicic et al, 2015).

DAB (3,3'-Diaminobenzidine) is an efficient marker of H_2O_2 accumulation *in vivo* (Thordal Christensen *et al.,* 1997) and can be reliably used for delineating stress response in crop plants. DAB assay has been suggested as an effective qualitative assessment of plant response to biotic and abiotic stress and measures the intensity of oxidative burst under stress. Since the oxidative burst is an early response to stress, in terms of production of reactive oxygen species (ROS) including hydrogen peroxide (Bindschedler *et al.*, 2006), the qualitative evolution can be differentially tracked in different parts of plant under stress to assess the most vulnerable part under stress. It is done by staining with 3,3'-diaminobenzidine (DAB) which is oxidized by hydrogen peroxide and generates a dark brown precipitate. In this paper the use of DAB assay as an efficient qualitative screen for stress tolerance is validated using common bean and cowpea.

MATERIAL AND METHODS

Plant Material

Fifty genotypes of common bean (47 breeding lines and three released varieties namely SR-1, SFB-1 and Arka Anoop. While the SR-1 and SFB-1 have been released by SKUAST-Kashmir, Arka Anoop has been released by IIHR, Bengaluru) and 40 genotypes of cowpea (39 landraces collected from different areas of the valley and one released variety *viz.*, Shalimar Cowpea-1 released by SKUAST-K) were evaluated in the present study in the greenhouse facility of Faculty of Agriculture, SKUAST-K, Wadura. Each genotype was grown in PVC columns of 1.2 m height and 20 cm diameter. The genotypes used were selected on the basis of their performance in the yield screening trials and represented diverse market classes in terms of use category, growth habits and seed characteristics.

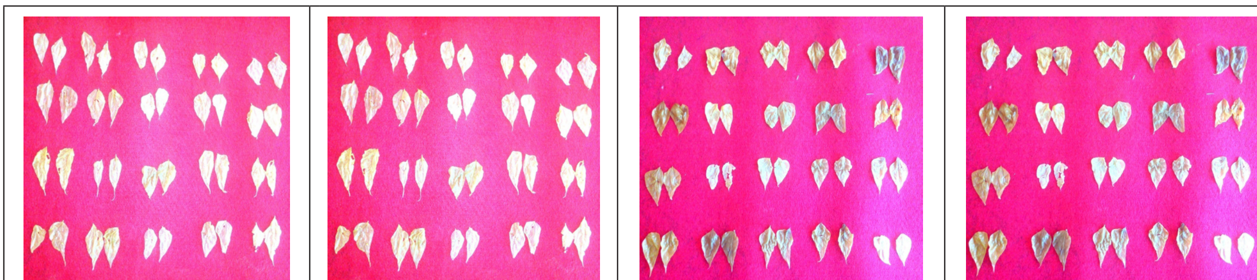
Methodology used for DAB assay

DAB assay was done as per Daudi and O'Brien (2012). In this protocol, the *in situ* detection of hydrogen peroxide (one of several reactive oxygen species) is done by staining with 3,3'-diaminobenzidine (DAB). DAB is oxidized by hydrogen peroxide in the presence of some haem-containing proteins, such as peroxidases, to generate a dark brown precipitate. This precipitate is exploited as a stain to detect the presence and distribution of hydrogen peroxide in plant cells. DAB staining solution was prepared by adding 50 mg DAB and 45 ml sterile H_2O for a final 1 mg ml^{-1} DAB solution in a 50 ml falcon tube. The tube was covered with aluminum foil as DAB is light-sensitive. About 25 ìl Tween 20 (0.05% v/v) and 2.5 ml 200 mM Na_2HPO_4 was added to the DAB solution to produce 10 mM Na_2HPO_4 DAB staining solution. Fully opened leaves of comparable size were selected from each treatment and incubated for one hour in falcon tubes with 2 ml of the DAB staining solution with the volume being adjusted to ensure that leaves were immersed. All the falcon tubes from both drought and irrigated treatments were shaken for 4-5 h at 80-100 rpm. Following the incubation, the aluminum foil was replaced and the DAB staining solution replaced with bleaching solution (ethanol : acetic acid : glycerol in ratio of 3:1:1). The falcon tubes were put in a boiling water bath (~90-95 °C) for 15 min. This bleaches out the chlorophyll but leaves the brown precipitate formed by the DAB reacting with the hydrogen peroxide. The time was adjusted (± 5 min) depending on the appearance of the leaves (they should be completely devoid of chlorophyll). After 15 ± 5 min of boiling, bleaching solution was replaced with fresh bleaching solution and allowed to stand for 30 min. Leaves were directly visualized for DAB staining. Photographs were taken under uniform lighting.

RESULTS AND DISCUSSION

In our studies on use of DAB assay for stress screening, staining was done for all the 50 common bean and 40 cowpea genotypes under irrigated and drought conditions. In both the crops, the assay clearly differentiated the lines on the basis of darker staining of leaves under drought. The lines showing greater per cent reduction in yield parameters show greater staining in DAB assay underlining the reliability of using this assay as a reliable supplement to phenotyping protocols for characterizing large germplasm sets. The assay is cost effective, less time consuming and can be replicated across crops as compared to spectophotometric methods for various reactive oxygen species. The method can be suitably modified to create quantitative data sets using freely available image processing softwares and apps such as ImageJ, Color Grab or Color Picker etc. In both common bean and cowpea, not many reports of using DAB assay is documented although recently various reports have come from *Arabidopsis* (Ding *et al.*, 2015) and tobacco (Chen *et al.*, 2016) especially in transgenic experiments. All these studies reported utility of DAB staining assay for differentiating wild type and transgenic plants containing various genes that enhance antioxidant activity. In such experiments, horseradish peroxidase (HRP) that catalyses oxidation of DAB by H_2O_2. The location of HRP can be visualized by light microscopy through formation of brown precipitates (Jovicic et al, 2015).

In terms of various physio-biochemical parameters compared for a set of two tolerant and susceptible lines each in common bean and cowpea (Table 1), it was observed that all the parameters corresponded well with the DAB staining assay except chlorophyll stability index (CSI) in common bean as well as cowpea. However, not many differences could be observed for relative water content as well as CSI in cowpea. This is possibly due to the fact that antioxidant systems are not significantly implicated in stress response in cowpea (Cavalcanti *et al.*, 2004). However in other crops H_2O_2 has been reported to initiate localized oxidative damage in leaf cells leading to disruption of metabolic function and loss of cellular integrity, actions that result in senescence (Omae *et al.*, 2005).



SKAU-WB-341	SKAU-WB-1634	SKAU-WB-6	SR-1
DAB profile of Tolerant common bean lines		**DAB profile of Susceptible common bean lines**	

C-18	C-25	C-2	C-12
DAB profile of Tolerant cowpea lines		**DAB profile of Susceptible cowpea lines**	

Table 1. Comparative performance of tolerant susceptible cultivars for various physio-biochemical parameters under stress in common bean and cowpea

Parameter	Common bean				Cowpea			
	Susceptible lines		Tolerant lines		Susceptible lines		Tolerant lines	
	SKAU-WB-6	SR-1	SKAU-WB-341	SKAU-WB-1634	C2	C12	C18	C25
Canopy temperature depression (°C)	0.71	-0.49	1.15	2.74	1.83	-1.00	0.78	0.27
Relative water content (%)	55.38	55.78	67.89	73.97	69.67	67.61	58.53	77.30
Membrane stability index	0.36	0.22	0.68	0.59	0.27	0.43	0.45	0.48
Chlorophyll stability index	0.86	0.26	0.82	0.78	0.78	0.84	0.54	0.62
Proline content (µmol/g)	31.23	24.42	31.42	31.12	25.26	19.28	41.17	31.61

The detection of cellular levels of H_2O_2 using DAB staining method and our results shows a clear difference in the degree of staining achieved in the stressed plant. Under drought stress, there was significant variation in staining in different genotypes indicating differential oxidative damage on account of production of H_2O_2 under stress like other reactive oxygen species (e.g. $O_2^{\cdot-}$, $OH^{\cdot}$, $^1O_2$) that are partially reduced or activated forms of atmospheric oxygen (Choudhury et al, 2017). The lines which showed fair amount of tolerance to drought in terms of higher yield and lower reduction had almost negligible staining while as the genotypes which showed lower yield showed higher reduction, distinctly darker staining. However, DAB is only a qualitative test for evolution of reactive oxygen species such as H_2O_2 and the genotypes showing greater staining under drought can be further analyzed for the amount of H_2O_2 through various analytical methods.

Under drought stress conditions, plants accumulate reactive oxygen species (Verslues *et al*., 2006). Through partially reduced or activated derivatives of oxygen, ROS can destroy DNA, proteins and carbohydrates, resulting in cell death (Mittler *et al*., 2004). Jiang *et al*. (2016) reported that DAB staining analyses can effectively discriminate the ROS levels in transgenic lines of rice with lower staining in transgenic lines as compared to control plants after drought-stress treatment. Chakraborty and Pradhan (2012) also reported that DAB assay was in conformity with results obtained on the basis of membrane stability and other biochemical parameters in tolerant and susceptible wheat varieties. However, in the present study, only qualitative assessment of DAB was done, the reliability of DAB assay can be further established by undertaking quantitative assessment of hydrogen peroxide evolution under water stress in common bean and cowpea.

The detection of cellular levels of H_2O_2 was done by DAB staining method and our results shows a clear difference in the degree of staining achieved in the stressed plant. Under water stress, there was significant variation in staining in different genotypes indicating differential oxidative damage on account of production of H_2O_2. The lines which showed fair amount of tolerance to water stress in terms of higher yield and lower reduction had almost negligible staining while as the genotypes which showed higher reduction had distinctly darker staining.

Evidences of DAB assay efficiency from other crops

Even though DAB assay has been routinely used in histochemical analyses in medical field, recently it has also found increasing use in crop plants especially in various transgenic research for stress tolerance to assess the ROS driven oxidative damage (especially H_2O_2). Among legume crops DAB assay has been reported for use in common bean, cowpea, mungbean, *Medicago*, *Lotus* etc. The published records about use of DAB assay for quantifying the ROS driven oxidative damage caused under biotic and abiotic stress.

Table 2. Published records of use of DAB assay for stress screening in crop plants

Crop	Stress	Reference
Medicago trancatula	Biotic stress (fungi)	Lanfranco et al (2005)
Lotus japonicas	Biotic stress (fungi)	Lanfranco et al (2005)
Rice	Drought stress	Ouyang et al, 2010
Tomato	Drought stress	Loukehaich et al, 2012
Ponicuria trifoliate	Drought stress	Huang et al, 2013
Arabidopsis	Drought stress	Ding et al, 2015
Tobacco	Drought stress	Sun et al., 2015
Rice	Drought stress	Jiang et al., 2016
Common bean	Drought stress	Sofi et al, 2017
Mung bean	Cold stress	Chen et al, 2017
Rice	Salinity stress	Basu et al., 2017
Maize	Drought stress	Munezeh et al., 2018
Common bean	Drought stress	Asmat Ara, 2019

Cowpea	Drought stress	Gull et al., 2019
Juglans regia	Drought stress	Yong et al., 2019
Quercus acutissima	Drought stress	Yong et al., 2019
Cedrus obusta	Drought stress	Yong et al., 2019
Tobacco	Drought stress	Zhao et al., 2020

Disadvantages of DAB assay

Despite being consistent in reliability, DAB is a qualitative assay foe histochemical peroxide evolution under stress. A major disadvantage of DAB assay is that it reacts slowly with hydrogen peroxide, even without the presence of a peroxidase enzyme. This means that once the DAB is combined with hydrogen peroxide, it should be used relatively quickly to avoid generating brown precipitate in the solution, which will contribute to background staining. Typically, kits for DAB staining are supplied with concentrated DAB substrate, and a separate buffer containing hydrogen peroxide, to be combined shortly before use. Another major issue pertains to handling, as DAB is reported to be toxic.

CONCLUSION

Plant breeders invariably deal with large germplasm sets for screening for stress resilience. As such reliable, easy to use and high throughput methods are an imperative. DAB assay provides an efficient screening tool especially when dealing with large germplasm sets to identify genotypes that undergo rapid oxidative damage under stress. The protocol can be used across diversity of crops and can be suitably modified for quantitative estimation of oxidative damage under stress.

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