

Reactive Oxygen Species (ROS) Accumulation in Yam Leaf Tissues and Field Screening as an Indicator of Yam Anthracnose Disease (YAD) Susceptibility

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Abstract

*Plant exposure to pathogens often triggers oxidative stress, marked by the accumulation of reactive oxygen species (ROS), particularly hydrogen peroxide (H₂O₂). In this study, we investigated the oxidative stress response of six yam (*Dioscorea* spp.) landraces of Cross River origin when challenged with yam anthracnose disease (YAD), caused by *Colletotrichum alatae*. Six yam landraces were arranged in a Randomized Complete Block Design (RCBD) with three replications in a field trial using minisetts planted under natural anthracnose pressure to evaluate disease susceptibility. Disease severity was scored using a standardized scale, and resistance levels were inferred from symptom expression. To assess oxidative stress, 3,3'-Diaminobenzidine (DAB) staining assays were performed on leaf samples to visualize H₂O₂ accumulation. The differential oxidative stress responses among landraces suggest that H₂O₂ accumulation correlates with susceptibility to YAD. Yeibo exhibited minimal oxidative stress, with low H₂O₂ accumulation and showed strong resistance to YAD. Okpolukata and Esange displayed intermediate H₂O₂ levels, suggesting a balanced oxidative response. Results from the field showed that Esange was resistant, while Okpolukata was susceptible to YAD. Obuna Otayi Kabor and Efala showed high H₂O₂ accumulation and were highly susceptible to YAD. Ija demonstrated fluctuating H₂O₂ levels but was classified as resistant under field conditions. Landraces like Yeibo, with lower oxidative stress, may possess more efficient antioxidant defense systems, contributing to better growth and yield. In contrast, excessive ROS in susceptible landraces may impair physiological functions, reducing productivity. Here, we show that hydrogen peroxide (H₂O₂) was produced in susceptible yam landraces subjected to YAD under natural field conditions.*

1.0 Introduction

Yams (*Dioscorea spp.*) are monocotyledonous plants with underground tubers and constitute the predominant starchy staple in sub-Saharan Africa especially in the five West African countries (Nigeria, Cote d'Ivoire, Ghana, Benin, Togo) which are widely regarded as the 'Yam belt region' (Ntui *et al.*, 2021). There are about 600 known species of yam, the most important edible species are *D. rotundata* (white yam), *D. alata* (water yam), *D. cayenensis* (yellow yam), *D. dumetorum* (bitter yam), *D. esculenta* (Chinese yam), and *D. bulbifera* (aerial yam). Among these six species, the white yam (*Dioscorea rotundata*) commands the highest market value owing to the superiority and preference of its tubers for food in Nigeria (FAO, 2013).

White yam plays crucial role as a food source and in the socio-cultural life of millions of people in West and Central Africa (Delga, 2020; Ekwere *et al.*, 2023). It is a staple crop that has contributed enormously to the subsistence of these populations. The tubers are rich in carbohydrates and provide a source of energy for a large segment of the population in these regions (Morse, 2021). Additionally, yams have cultural significance and are often featured in festivals and ceremonies such as the *Leboku* festival in Cross River State, symbolizing wealth and prosperity (Eja and Violet, 2015).

They are important not only significant for their nutritional value but also their medicinal properties such as antioxidant, anti-obesity and immunomodulatory properties which help in the maintenance of proper physiological functions (Tareen *et al.*, 2025). Recent research has focused on determining reactive oxygen species (ROS) in plant tissues, as these molecules play a crucial role in plant stress responses and signaling pathways (Shareen *et al.*, 2023). Like other green plants, the leaves of yams are exposed to environmental stressors that can induce the production of reactive oxygen species. These stressors include pathogen attack, drought, salinity, and extreme temperatures (Romy *et al.*, 2016).

Reactive oxygen species (ROS) are highly reactive metabolites of oxygen derived as by-products of aerobic activities in living organisms (Das and Roychoudhury, 2014). While over-production of ROS can be toxic to cells, at lower concentrations, they can mediate signaling pathways that facilitate tolerance against environmental stress (Das and Roychoudhury, 2014; Li *et al.*, 2022).

ROS act as signaling molecules at lower concentrations, mediating various physiological processes. They are involved in activating defense genes and regulating plant hormones that control growth and development (Rai, 2023). This signaling function is particularly crucial when plants face environmental stressors such as drought, salinity, extreme temperatures, and pathogen attacks. The ability of ROS to signal such stress allows plants to activate their defense mechanisms promptly, facilitating adaptation and survival (Prakash *et al.*, 2021; Lukan and Coll, 2022).

However, the overproduction of ROS can lead to oxidative stress, a state where the accumulation of ROS can cause cellular damage. This damage includes the peroxidation of lipids, denaturation of proteins, and mutations in DNA, which can ultimately lead to cell death (Zeng and Chen, 2024). To counteract this, plants have evolved a sophisticated antioxidant defense system. This system comprises both enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), and peroxidases, and non-enzymatic antioxidants, including ascorbic acid, glutathione, and various phenolic compounds. These antioxidants work synergistically to scavenge excess ROS, thus protecting the cell from

oxidative damage and ensuring the maintenance of redox homeostasis (Ahmad *et al.*, 2019; Zeng and Chen, 2024).

Research has shown that the antioxidant capacity of yams is not only important for their resilience against environmental stress but also for their nutritional value. The antioxidants present in yams such as phenols, flavonoids and anthocyanins can contribute to the good health as they are known to combat oxidative stress in humans (Padhan *et al.*, 2020; Adomėnienė and Venskutonis, 2022).

ROS are key indicators of plant stress and can influence yam crops' growth, health, and productivity. By understanding the levels and behavior of ROS, researchers can identify yam varieties that are more resilient to environmental stresses and diseases. This work sought to (a) profile hydrogen peroxide (H_2O_2) accumulation as an indicator for reactive oxygen species (ROS) in leaf tissues of six yam landraces under natural anthracnose disease pressure (b) to correlate oxidative stress levels with anthracnose susceptibility and identify resistant genotypes

2.0 Materials and Methods

2.1 Study Area

The field screening of the yam plants was carried out at the Research Farm of the Department of Genetics and Biotechnology, University of Calabar, Nigeria. Six *Dioscorea* landraces - *Yeibo*, *Okpolukata*, *Obuna Otayi kabor*, *Ija*, *Esange* and *Efala* were selected randomly for field evaluation based on farmer preference in Cross River State. These landraces represent the most grown yam landraces across the State. The experiment was conducted under natural anthracnose disease pressure during the peak rainy season using the Randomized Complete Block Design (RCBD) with the six landrace treatments in three replications.

2.2 Sample Collection

Leaf samples of *Yeibo*, *Okpolukata*, *Obuna Otayi kabor*, *Ija*, *Esange*, and *Efala* were obtained from the Research farm of the Department of Genetics and Biotechnology, University of Calabar, Cross River State. The leaves were collected at six weeks after emergence, when the leaves are young and early onset of anthracnose symptoms began to appear. This stage typically included young, expanding leaves, which are more physiologically active and can give a good indication of disease resistance. Leaves at this same stage which are sensitive to oxidative stress was also used to assay for ROS accumulation. The leaf assay experiment was conducted at the Laboratory of Plant Tissue Culture and Molecular Biology of the Department of Genetics and Biotechnology, University of Calabar.

2.3 Methods

The adaptation of the previous method developed by Thordal-Christensen *et al.*, (1997) was used for the DAB assay for the detection of hydrogen peroxide (H_2O_2). DAB known chemically as 3,3'-Diaminobenzidine is a laboratory technique used for the detection of hydrogen peroxide (H_2O_2) in plant tissues. It offers a visual cue of the level of oxidative stress a plant is undergoing. DAB reacts with hydrogen peroxide in the presence of natural

plant enzymes such as peroxidases to form a brown-coloured precipitate. The darker the brown stain on the leaf, the more hydrogen peroxide is present, and this is indicative of higher oxidative stress.

2.4 Field Screening for Disease Resistance

The field screening was done using plants that were freely exposed to the yam anthracnose disease (YAD) occurring naturally. The disease symptoms were recorded at 24 Weeks after planting (WAP) when over 80 % of the plants presented symptoms. Data was collected on number of leaves per plant, number of infected leaves per plant. Disease incidence (DI %) was determined per landrace using the formula:

$$DI (\%) = \text{Number of Infected Leaves} \div \text{Total Number of Leaves} \times 100 \dots \dots \dots \text{Equation 2.1}$$

and Disease severity (DS %) was calculated according to the modified method of Manadhar *et al.* (2016) as:

$$\text{Damage Index (DI)} = (\text{Disease Incidence} \times \text{Severity}) / \text{Highest Severity Class} \dots \dots \text{Equation 2.2}$$

Then, Disease severity index (DSI) was calculated as:

$$DSI = \text{Disease Incidence} \times \text{Disease Severity} \dots \dots \dots \text{Equation 2.3}$$

The results from these were used for the classification into various resistance levels. This was done using the five-point scale range of Aduramigba – Modupe *et al.* (2014) as follows:

1	0 – 1 %	Highly Resistant (HR)
2	2 – 10 %	Resistant (R)
3	11 – 25 %	Moderately Resistant (MR)
4	26 – 50 %	Susceptible (S)
5	50 – 100 %	Highly Susceptible (HS)

3.0 Results and Discussion

3.1 Field Screening for YAD Resistance

The Disease Severity Index (DSI) was used to assess resistance to YAD. Landraces with a DSI ranging from 2% to 10% were classified as resistant (R), while those with a DSI between 50% and 100% were categorized as highly susceptible (HS). Among the tested landraces, *Yeibo*, *Ija*, *Esange*, and *Efala* exhibited resistance (R), whereas *Okpolukata* and *Obuna Otayi Kabor* were highly susceptible (HS) (Table 3.1).

Notably, all the resistant landraces except *Efala* belonged to the *Dioscorea rotundata* species, while the highly susceptible ones were classified under *Dioscorea alata*. The HS group showed symptoms indicative of oxidative stress, whereas the R group displayed resistance, suggesting a potential difference in their stress response mechanisms.

3.2 Oxidative Stress Assessment Using the DAB Assay

The DAB assay revealed varying levels of oxidative stress in different yam leaf samples, as indicated by differences in brown staining intensity. Each yam variety exhibited distinct staining patterns, reflecting varying concentrations of hydrogen peroxide (H_2O_2). The results presented in Table 3.1 and Figures 3.1(a–f) demonstrated significant differences in oxidative stress levels among the yam varieties, with more intense brown staining correlating with higher H_2O_2 accumulation.

Yeibo yam leaves exhibited minimal oxidative stress, as evidenced by light brown staining in the DAB assay. This indicates low H_2O_2 production, likely due to an efficient antioxidant defense system that effectively scavenges reactive oxygen species (ROS) and maintains cellular homeostasis. The reduced H_2O_2 levels in *Yeibo* yams suggest a robust enzymatic antioxidant response and limited exposure to environmental stressors and pathogens.

In contrast, *Okpolukata* yam leaves showed moderate brown staining, indicating intermediate H_2O_2 accumulation and a corresponding level of oxidative stress.

Table 3.1: Field screening for YAD resistance in 6 yam landraces at 24 WAP

S/N	Landrace	No. of infected leaves	Total no. of leaves	Disease incidence (%)	Disease severity (%)	Disease severity index	Rating
1	<i>Yeibo</i>	71	339	20.94	0.46	9.63	R
2	<i>Okpolukata</i>	661	1349	49.00	1.60	78.4	HS
3	<i>Obuna otayi kabor</i>	349	478	73.01	1.26	91.99	HS
4	<i>Ija</i>	69	847	8.15	0.23	2	R
5	<i>Esange</i>	18	1710	1.10	1.27	1.40	R
6	<i>Efala onere</i>	16	179	8.94	0.22	2.0	R

Note: WAP-Weeks after planting, YAD- Yam anthracnose disease

Okpolukata, *Obuna Otayi kabor* and *Efala* yam leaves showed intense brown staining in the DAB assay, which signified high levels of H_2O_2 production and significant oxidative stress. This level of oxidative stress indicated that trio of *Okpolukata*, *Obuna Otayi kabor* and *Efala* yams were experiencing oxidative stress due to YAD.

This result aligns with the findings of Luna *et al.* (2011), who associated high oxidative stress levels with environmental stressors or metabolic imbalances in plant tissues. However, as Veljovic-Jovanovic *et al.* (2002) noted, phenols can interfere with H_2O_2 detection, meaning the actual oxidative stress in these leaves may be even higher than indicated by the staining. This finding is comparable to the study on wheat leaves by Liu *et al.* (2014), where plants with significant oxidative stress exhibited a well-coordinated antioxidant response.

Ija leaves exhibited varying degrees of brown staining in the DAB assay, ranging from light to moderate. This variability indicates fluctuating levels of H_2O_2 production and oxidative stress, reflecting different physiological responses or environmental conditions. The inconsistency in stress levels can be attributed to variable exposure to pathogens and fluctuating antioxidant defense. This variability indicates fluctuating levels of H_2O_2 production and oxidative stress. Wu *et al.* (2019) reported similar findings, emphasizing the significant impact of environmental factors on ROS production and plant antioxidant responses. The presence of phenols has contributed to the observed variability by scavenging ROS, leading to inconsistent detection of H_2O_2 levels.

Table 3.2: Oxidative stress levels observed in different yam landraces

Yam landrace	DAB Assay Staining Intensity	Interpretation
<i>Yeibo</i>	Light brown staining	Low ROS Level, minimal oxidative stress
<i>Okpolukata</i>	Intense brown staining	High ROS levels, significant oxidative stress
<i>Obuna otayi kabor</i>	Intense brown staining	High ROS levels, significant oxidative stress
<i>Ija</i>	Light to moderate brown staining	Varying ROS levels (low to intermediate), varying degrees of oxidative stress
<i>Esange</i>	Moderate brown staining	Intermediate ROS levels, moderate oxidative stress
<i>Efala</i>	Intense brown staining	Low ROS levels, minimal oxidative stress

Esange yam leaves showed moderate brown staining in the DAB assay, close to *Yeibo*, indicating intermediate levels of H_2O_2 production and moderate oxidative stress. This indicated that *Esange* and *Yeibo* yams have a comparable antioxidant response to maintain cellular stability under YAD stress conditions. This result aligns with the study by Wang *et al.* (2020), where an efficient antioxidant system effectively scavenged reactive oxygen species (ROS), maintaining cellular health. Varghese *et al.* (2014) observed a comparable antioxidant response in human tissue, where moderate oxidative stress levels activated signaling pathways enhancing antioxidant defenses. As with other yam varieties, the phenols present in *Esange* and *Yeibo* yam leaves may have played a role in mitigating the full detection of oxidative stress and this could mean that the actual oxidative stress could be slightly higher than observed.

The study by Ruifrok and Johnston (2001) on the comparison of quantification of histochemical staining by hue-saturation-intensity (HSI) transformation and colour deconvolution emphasized the importance of accurately detecting H_2O_2 to understand oxidative stress dynamics. It is important to note that the determination of H_2O_2 in plant tissues is influenced by various factors, including phytochemicals such as phenols. According to Veljovic-Jovanovic *et al.* (2002), phenols can interfere with the accurate detection of H_2O_2 due to their antioxidant properties and ability to scavenge free radicals, potentially leading to an underestimation of oxidative stress levels.

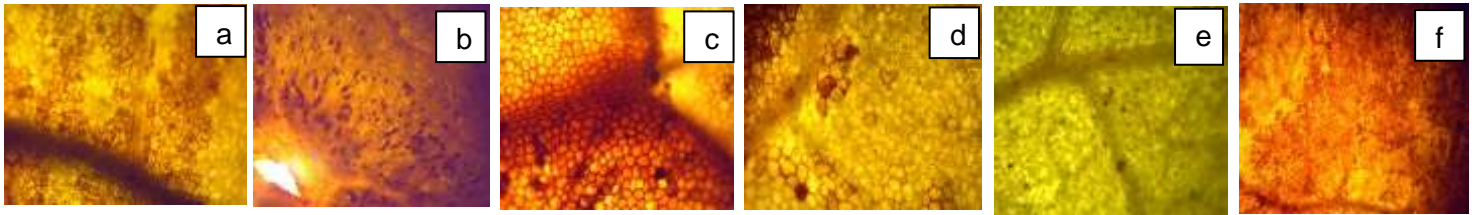


Figure 3.1 Photomicrograph showing variation in oxidative stress in yam leaves. a-f indicate 6 landraces (a) *Yeibo* – light brown staining (b) *Okpolukata* – intense brown staining (c) *Obuna Otayi kabor* – intense brown staining (d) *Ija* - Light to moderate brown staining (e) *Esange* - Moderate brown staining (f) *Efala* - Intense brown staining

The results of this study showed that field screening for disease susceptibility to YAD is not sufficient on its own. The utilization of DAB assay for the detection of ROS in each of the susceptibility/resistant landraces was important to corroborate the results of the field screening. These results, when taken together, indicate that while ROS (H_2O_2) serves as a signaling molecule, its presence is implicated in YAD conditions and could serve as a rapid diagnostic procedure for plant disease indication in yam fields. The variation in the production of H_2O_2 in the different landraces is indicative that the procedure can discriminate the different yam landraces based on their susceptibilities to the YAD.

4.0 Conclusion/Recommendation

The present study investigated the relationship between reactive oxygen species (ROS) accumulation - specifically hydrogen peroxide (H_2O_2) - and anthracnose susceptibility in six yam (*Dioscorea* spp.) landraces under natural field conditions. Using 3,3'-diaminobenzidine (DAB) staining, it was demonstrated that landraces exhibiting lower levels of H_2O_2 (*Yeibo*, *Esange*) consistently showed reduced disease symptoms, whereas those with elevated ROS production (*Obuna Otayi Kabor*, *Okpolukata*) showed increased susceptibility. These findings affirm the utility of H_2O_2 as a physiological biomarker for evaluating genotype resilience to yam anthracnose disease (YAD). The correlation between ROS accumulation and field resistance screening strengthens the case for the use of biochemical screening tools in disease - resilience breeding programs. Such collective approaches link molecular diagnostics with agroecological performance, thus, accelerating the identification and propagation of tolerant/resistant landraces.

Based on the conclusions, the following recommendations were made: The incorporation of DAB staining assays into pre-field screening activities of yam breeding programmes to facilitate early detection of oxidative stress and predict varietal susceptibility to YAD,

selection and in vitro multiplication of resistant landraces, such as *Yeibo* and *Esange*, in breeding programmes aimed at anthracnose mitigation.

In addition, ROS-based assays should be expanded to include enzymatic antioxidant profiling such as catalase, peroxidase, superoxide dismutase, thereby generating more knowledge of underlying plant defence mechanisms in YAD. Multi-location trials should be carried out to validate the performance of landraces and ROS - disease correlations across the different agroecological zones of Cross River State. Molecular markers associated with ROS homeostasis and anthracnose resistance should be developed for application in marker-assisted selection (MAS) strategies.

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