# ACTIVITIES OF OXIDATIVE ENZYMES OF BOTH HEALTHY AND FUNGI-INFECTED CASSAVA ROOTS

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## ABSTRACT

*Production of oxidative enzymes of post-harvested cultivars of cassava was investigated in the laboratory when inoculated with four pathogenic fungi causing rot disease. Three cultivars of cassava namely Oko-Iyawo; Odongbo (local cultivars); and TMS 4(2) 1425 (a hybrid cultivar) and four pathogenic organisms (Fusarium pallidoroseum; Macrophomina phaseolina; Lasiodiplodia theobromae; and Rhizopus stolonifer) were used in this study. Results of this study revealed that Polyphenol oxidase (PPO) and Peroxidase activities increased from day 1 (one) up to day 6 (six) or 8 (eight) in both healthy and infected cultivars. Regression analysis revealed a measure of rate of change in infection caused by different test organisms as induced by storage days. Mycelia dry weights of the test pathogens decreased with decreasing incubation periods. Enzyme production and activities of test pathogens in the utilization of different carbohydrate sources varied with the different carbohydrate sources and the test pathogens.*

*Generally, this study showed that post-harvest rot disease of cassava roots is widely distributed and that presence of the oxidative enzymes within the harvested roots could not totally impose resistance against ingress of the pathogens. They can only create immunity against these pathogens for a short time; this was according to the regression analysis, which shows that the shelf-lives of the roots depend largely on the number of storage days and the varieties of the cassava roots after harvest. Thus, measures to increase the shelf life of harvested cassava roots should be taken into consideration.*

Keys: Post-Harvest, Oxidative enzymes, Pathogen, Shelf-life.

## INTRODUCTION

Cassava (Manihot esculenta Crantz) is a woody shrub that grows mainly in tropical lowland areas, although it is also cultivated in tropical highlands and subtropical areas. It originated from north-east Brazil with the likelihood of an additional center of origin in Central America (Adewolu, 1999). Cassava is planted in several regions as a security crop to avoid famine after a drought season due to the loss of other crops

(IITA, 1995). It is also a very important cash crop in South-East Asian countries, where most of the production is exported to developed countries as pellets for animal feed. The world production of cassava is estimated at 163 million tonnes of fresh roots with an average yield of 10tonnes/ha. Nigeria, Brazil, Thailand, Zaire and Indonesia are the major producers (FAO, 2000). It is interesting to observe the fast growth of cassava production in both Asia during 1972-1980 and in Africa during the last 10 years. The plant can be grown on marginal land of alfisol type of soil with low pH and a high concentration of soluble aluminum (Wenham, 1995). All these are characteristics of depleted tropical soils that are unsuitable for the majority of other crops. Although the optimum conditions for growth are in the sub-humid and humid tropics with annual rainfall of 1500 mm, cassava is drought tolerant and can be grown in locations with less than 600 mm of precipitation and erratic rainfall where the dry season lasts as long as eight months (El-Sharkawy, 1993). Cassava is one of the most important tropical crops being the main source of calories for more than 400 million people in Africa and probably world wide (Cock, 1985) . A survey in Ghana reported that 30% of the cassava farmers mention perishibility of the roots as a major risk in cassava production (Wenham, 1995). There is also a decrease in the quality of the roots by reduction of their starch content, and an increase in fiber and cooking time (Rickard and Coursey, 1981). According to Booth (1975) there are two types of post-harvest deterioration in cassava. The primary or physiological deterioration which is the initial cause of loss of acceptability of roots for fresh consumption is identified by fine blue-black streaks in the root vascular tissue. The secondary or microbial deterioration of the roots occurs when the roots have already become unacceptable due to the primary deterioration (Booth, 1975). It is caused by pathogenic rots, fermentation and/or softening of the roots. Several microorganisms have been associated with microbiological deterioration, including *Aspergillus flavus, Fusarium solani, Botryodiplodia theobromae, Trichodema harzianum,* among others (Oluma, 1992). These microorganisms can generally be isolated at 7days post-harvest, after signs of physiological deterioration have become apparent.

The major problem with the crop is post-harvest root deterioration that occurs a few days after harvest usually caused by some pathogens that can cause significant yield losses. One of the biochemical mechanisms involved in this problem is the production of several secondary plant products originating from or passing through the internal systems via different pathways of the infected post-harvested root tuber (Wenham, 1995). Polyphenol oxidase and Peroxidase enzymes are critical enzymes in one of these pathways, working as a trigger for the production of other secondary compounds (Douglas et al., 1991; van de Meer et al., 1993). These enzymes are parts of plants defense against many different stresses and an increase of their activities in plants under different stresses have been extensively reported (Jones, 1984). Since these enzymes are part of the biosynthetic pathways for diverse plant natural products, studies on them are important in understanding the defense and the wound response mechanisms in plants. This can also be used as a model to study the basic mechanisms of different activities and responses of plants to infections by pathogens (Dixon and Paiva, 1995). They are also intrinsically related to how plants respond to stresses, and play a key role in the protective mechanism of the plant. An increase in their activity occurs during both incompatible plant pathogen interactions and wounding of plant tissues. These enzymes participate in the oxidation of phenolic compounds which are the products originating from their activities that will be cross-linked in the cell wall to form lignin and suberin (Ni et al., 1996). Plants employ a variety of defense mechanisms during a resistance response to pathogens. These mechanisms can work as a cascade of events or simultaneously depending on the stage of the interaction and the defensive

strategy used (Campa, 1991). Generally the defense mechanisms include the use of mechanical barriers, defensive proteins and defensive enzymes. Polyphenol oxidase and peroxidases are enzymes involved in the oxidation of compounds (Jalali and Bhargava, 2002). They play a key role in several aspects of plant physiology and development, such as lignification and suberization of cell walls, IAA oxidation and post-harvest deterioration of fruits and vegetables (Popoola, 2006). An increase in their activity generally occurs after wounding, incompatible pathogenic interactions and physiological stress such as salinity, radiation, and pollution (Campa, 1991). In the case of both post-harvest root deterioration in cassava and the resistance interaction with pathogens, an increase in the oxidative enzyme pathway is expected beginning with an increase in their levels leading to an accumulation of phenolic compounds. In turn, oxidative enzymes will catalyse the oxidation of those phenolic compounds, producing structural barriers for the pathogens (Ni et al., 1996). Hence, this study investigates the activities of two oxidative enzymes of both healthy and fungi-infected harvested cassava roots.

## MATERIALS AND METHOD:

Collection of local samples was done in some local farms in various parts of Oyo State, Nigeria and also in International Institute of Tropical Agriculture (IITA) for the hybrid variety used for this study. They are: Oko-Iyawo; Odongbo (local cultivars); and TMS 4(2) 1425 (a hybrid cultivar). Both the healthy and the infected were carefully collected into sterilized polyethylene bags before bringing them to the laboratory for the various analyses. Pathogenic fungal organisms associated with the rot disease of cassava roots were isolated by excising the diseased samples; surface sterilized them with 1% Mercuric Chloride solution for Iminute before rinsing in several changes of sterilized distilled water. These were finally blotted dry in other to remove excess water. These specimens were also plated on Potato Dextrose Agar (PDA) and were incubated at 30oC for 24-72 hrs. On the emergence of fungal colonies, they were sub-cultured on freshly prepared PDA plates in order to obtain pure colonies while the slants were kept in McCartney bottles for future use. Pure mycelia colonies were teased and dropped into lacto phenol blue before identifying each of them under the compound microscope according to the standard test (Barnetts and Hunter, 1972). Standardized isolates of these organisms was established using Tuber Root Improvement Programme (TRIP) pathology laboratory, IITA, Ibadan, Nigeria and their level of infection was also established using the healthy roots collected before 10 days incubation. Rot indices ranging from 0-10 were also marked out to show the frequency of occurrence of the isolated pathogens.

### Enzyme Studies:

The oxidative enzyme studied in this work was done by preparing extracts from both the healthy and fungi-infected cassava roots. The activities of Polyphenol oxidase and Peroxidase enzymes were determined in fresh roots and in those stored at ambient conditions of 30oC for 10 days in all the three varieties used for this study. The extracts were centrifuged at 3000rpm for 10mins and the clear supernatant was precipitated with saturated ammonium sulphate. The precipitate was filtered and the filtrate further dissolved in respective buffers used for the study of the different enzyme assayed. Polyphenol oxidase activity was assayed following the procedure of Oluma (1992) and Adewolu (1999) with the extracts dissolved in 0.1M Phosphate buffer at pH 7.0. The reaction mixture contained 1ml of enzyme extract mixed with 1ml of 0.1M substrate (catechol) and 4ml of 0.1M phosphate buffer incubated for 5minutes and the absorbance was read on a Spectrophotometer at 495 nm. One unit of PPO activity was expressed as the amount of enzyme catalyzing an absorbance change of 0.1M in 5mins at 495nm under the

assay condition. Sterilized distill water was used as the extracting medium for control.
Peroxidase enzyme activity was also assayed following the modified method of Salami (1999). The reaction mixture contain 1 ml of extracts diluted with 10 ml of sterilized distill water and mixed with 1 ml of 0.1 ml of 0.1M Phosphate buffer at pH 6.5, 1 ml of 0.1M P-phenylenediamine, 1 ml of 0.1% Hydrogen peroxide (H2O2). The mixture was then incubated for 5 mins while 1 ml of sterilized distilled water was used as the extracting medium for control experiment. The absorbance was read at 495 nm using spectrophotometer where one unit of peroxidase activity was expressed as the amount of enzyme which catalyzed an absorbance change of 0.1M in 5mins at 495 nm under the assay condition.

**Effect of different carbohydrate sources on mycelia growth of fungal isolates and the enzyme activities.**
The carbohydrate sources used for this study are yam, cocoyam, sweet potato, irish potato, and cassava. Extracts of the different carbohydrate sources were prepared by boiling 250 g each of the carbohydrate sources for 1 hr in 1 L of distilled water. The supernatant was collected into 1litre measuring cylinder and made up to 1000 ml with distilled water. Each extracts was supplemented with 20g glucose. The mixture was then dispensed into 150 ml conical flasks and autoclaved at 121oC for 15mins, it was then allowed to cool down broths before inoculating with 5mm mycelial disc of the fungal isolate and replicated thrice. This process was repeated for all the four fungal organisms and the broths of all the carbohydrate sources. These were then incubated at 30oC for 7days, at the end of incubation period, the mycelia mats were harvested unto pre-weighed filter papers respectively and oven dried at 80oC for 24 hrs. They were then cooled in a dessicator and weighed. The dry weight of mycelia mats was obtained by subtracting the weight of filter paper from total weight.

## RESULTS

*Lasiodiplodia theobromae; Macrophomina phaseolina; Rhizopus stolonifer;* and Fusarium pallidoroseum were the fungal organisms studied causing rot disease of the three cultivars of cassava roots used for this study. Their inoculation into healthy roots of the same cultivars resulted initially in dark water-soaked regions at seven days after inoculation and subsequently to extensive necrosis around the infection areas (Table 1). All the fungi isolated from rotted cassava roots were found to be pathogenic even when inoculated into the healthy roots with their different characters and remarks (Table 2). Enzymes studied in this work are Polyphenol oxidase and Peroxidase enzymes.

Polyphenol oxidase (PPO) activity increased from day 1 (one) up to day 6 (six) in cultivars TMS 4(2)1425 and Oko-Iyawo with the peak of activity reached on day 6 while the peak of activity was reached on day 4 for cultivar Odongbo (Fig. 1A). PPO activity declined after the different peak days of activity for all the cultivars. In the healthy and infected roots, PPO activity took different pattern depending on the test pathogen. For instance, cultivar TMS 4(2)1425 inoculated with *F. pallidoroseum* had its peak of activity on day 8 of incubation while others had their peak of activities differently (Fig. 2A). Odongbo cultivar inoculated with *M. phaseolina* had its PPO activity on day 6 while the same cultivar inoculated with different test pathogen had it differently (Fig. 4A). Oko-Iyawo cultivar inoculated with *L. theobromae* had its peak of activity on day 6 while other test pathogens had theirs differently (Fig. 3A). Peroxidase enzyme activity was also observed in all the 3 cultivars (i.e. both healthy and infected harvested roots used in this study (Fig. 1B). Healthy cultivar TMS 4(2)1425 had its peak of peroxidase activity on day 4 and declined from the same day 6 while the same cultivar but infected had its peak of peroxidase activity between days 4 and 8 before the decline from day 8 (Fig. 2B). Cultivars Oko-Iyawo and

Odongbo (Fig. 4B) had their peaks of activity on day 4 followed by the decline of activity for both healthy and infected almost at the same time except Oko-Iyawo root inoculated with F. pallidoroseum (Fig. 3B). Regression analysis of the peroxidase activity reveals that the infected root treated with *L. theobromae* was least in reaching the peak on day 4 but fastest in declining from day 8 through to day 10, this was followed by R. stolonifer while *F. pallidoroseum* was fastest in reaching the peak of activity on day 4 (Figs 2B, 3B, and 4B). Effect of period of storage on the variation of polyphenol activity was found more on Odongbo cultivar followed by TMS 4(2)1425 but lowest in Oko-Iyawo with R2 value 0.4991 while it was found almost the same in peroxidase activity for all the three cultivars (Figures 1, 2, 3 & 4).

Mycelia dry weights of the test pathogens were found to be decreasing with the incubation periods. In PPO enzyme activity, *R. stolonifer* had the highest mycelia weight while *F. pallidoroseum* had the least (Fig. 5A). In Peroxidase enzyme activity, *L. theobromae* had the highest while *F. pallidoroseum* had the least mycelia dry weight. Enzyme production and activities of the test pathogens in the utilization of the different carbohydrate sources varied with the different carbohydrate sources at a highly significant level of less than 5% level of probability (Figs. 5A & B). All the test pathogens grew on the different carbohydrate sources which are yam, cocoyam, sweet potato, irish potato, and cassava. *L. theobromae* grew best on cocoyam, cassava and yam broths in descending order of utilization while the best growth of R. stolonifer was on Irish potato. *M. phaseolina* grew best on sweet potato and cocoyam broths while the best growth of *F. pallidoroseum* was on sweet potato, Irish potato, and cassava broths in descending order of utilization. *R. stolonifer* grew least in all the broths except on Irish potato broth (Figures. 5A & B). Peroxidase enzyme production of the test pathogen in different carbohydrate sources was found highest in L. theobromae grown on sweet potato and least in R. stolonifer grown on yam source (Fig. 5B). Whereas, PPO production was found highest in *R. stolonifer* grown on cocoyam and least in the same pathogen grown on both sweet potato and Irish potato (Fig. 5A).

## DISCUSSION

Hypersensitive reaction is a form of response put up by the host to prevent the spread of a pathogen (Agrios, 1998). This is often associated with the initiation of other responses, such as liginification and the synthesis of some antimicrobial compounds (Dickson and Lamb, 1990). This was observed in this study as a form of rapid active defense mechanism put up by host plant to prevent the spread of pathogenic infection. Also, the pathogenic indices and the remarks shown by the test pathogens during pathogenicity test of the rot infection of the cassava roots could have been a form of defense mechanism (Tables 1 & 2; Wolpert et al., 2002). This might have been elicited through the production of phytoalexins. They are low molecular weight antibiotics produced to inhibit the growth of pathogen during an infection and also to induce resistance against subsequent infections by the same pathogen (Agrios, 1998; Popoola, 2006). All these were observed in this study, though on very low concentrations (Figures.1, 2, 3, 4 & 5). This was when the peak and decline of oxidative enzyme activities of the healthy cassava root used was found to be common on day 6 while that of the infected roots was on different days ranging from day 4-8, this shows a level of hypersensitive reaction to pathogenic infection by the cassava roots in order to prevent the infection process (Figures 1, 2, 3 & 4). The activation of these oxidative enzymes during resistant interaction has been observed to provide several lines of defense for the cassava roots used especially the infected roots against the test pathogens. This is in line with the findings of Nagarathna et al. (1993) and Odebode et al. (2001). Formation of structural barriers in order to prevent the destructive actions of the test pathogens was discovered for

some days in this study to aid the catalytic activity of the oxidized peroxidase though at a low concentration. This was opined by Kolattukudy et al. (1992). Production of such barriers has also been reported by Boher et al. (1995) and Salami et al. (2001). Another function of these oxidative enzymes is in the area of plant defense initiated through the production of phytoalexins in form of antimicrobial activity especially the phenolic compounds to aid resistant interaction between the roots and the test pathogens. This was observed in this work as the different test pathogens attain their level of peak of activities and decline at different times given the barriers, resistance and defense been put up by the different cassava roots used. This corresponds with the findings of Kpemoua et al. (1996) and Dixon and Paiva (1995).

Period of storage also has effect on the activities of the oxidative enzymes. This was found through the regression analysis that healthy roots had high activities than the infected roots (Figures 1, 2, 3 & 4) and it is observed to be a form of plant-pathogen interaction and post-harvest deterioration which reveals similarities during the production of these oxidative enzymes and it is termed "a variation on a common theme" as opined by Baron and Zambrynski (1995) and Graham (1995). Days of storage also has effect on the accumulation of the phytoalexins as well as the production of the phenolic compounds. This was observed in both the healthy and infected as the regression analysis reveals that the activities of the oxidative enzymes were higher after some days of storage before the decline, this indicates the increased shelf-life of the cassava roots (Sato, 1993). Production of oxidative enzymes during cassava root post-harvest deterioration is expected since there is a preliminary increase in phytoalexins-produced phenolic compounds. The oxidative enzymes catalyse the oxidation of such phenolics to form a lignin layer on the infected surface (Plumbey et al., 1981; Tanaka, 1983; Wolpert et al., 2002). This thus, increases the shelf-life of cassava roots to an extent. This study reveals plant-pathogen interaction in the area of post-harvest deterioration of the roots. It also involves the oxidative enzymatic production by plants for both inhibition and resistance through the catalytic oxidation of phenolic compounds (by these oxidative enzymes) to form a layer of protection for the plant against the pathogen (Jalali and Bhargava, 2002).

Also, difference in the mycelia growth and weight of the different test pathogens suggests accumulation of phytoalexins like the phenolic compounds which could have been catalyzed by the presence of these oxidative enzymes (Mc Douglas, 1993) and Boher et al., 1995). Utilization of the different carbohydrate sources by the different test pathogens shows the strength of the test pathogens in degrading the tissues of the roots (Baron and Zambrynski, 1995; Ajiboye, 2004) and the extent of the strength of the oxidative enzymes in building resistance against these pathogens in their interactions (Pallas et al., 1996 and Agrios, 1998). Response of the cassava roots shows a form of variation on a common theme which in other words indicates a kind of defense mechanism put in place by the plant against pathogenic attack. This was also discovered by Baron and Zambrynski (1995); Iglesias et al. (1997); Jalali and Bhargava (2002). Oxidation of phenolic compounds was also observed in this work, since there were activities of the oxidative enzymes that usually cause the oxidation of the phenolic compounds which is not exclusive to defense mechanisms as well as the development of the plant (Graham, 1995).

Conclusively, this study has shown days of storage resulting in the accumulation of phytoalexins and that the resistance of cassava roots against pathogenic infection as well as the eradication of the physiological deterioration of the roots can be enhanced through the production of secondary metabolites. The catalysis of oxidative enzymes that will lead to

the production of phytoalexins which have antimicrobial activity was initiated in this plant during wounding and infection process. This led to the accumulation of these phytoalexins around the wound and the infection sites of the roots, which provided resistance against the pathogens and created a form of defense that increased the shelf-life of the root. This has also been opined by Kpemoua et al. (1996); Dixon and Paiva (1995) and Graham (1995).

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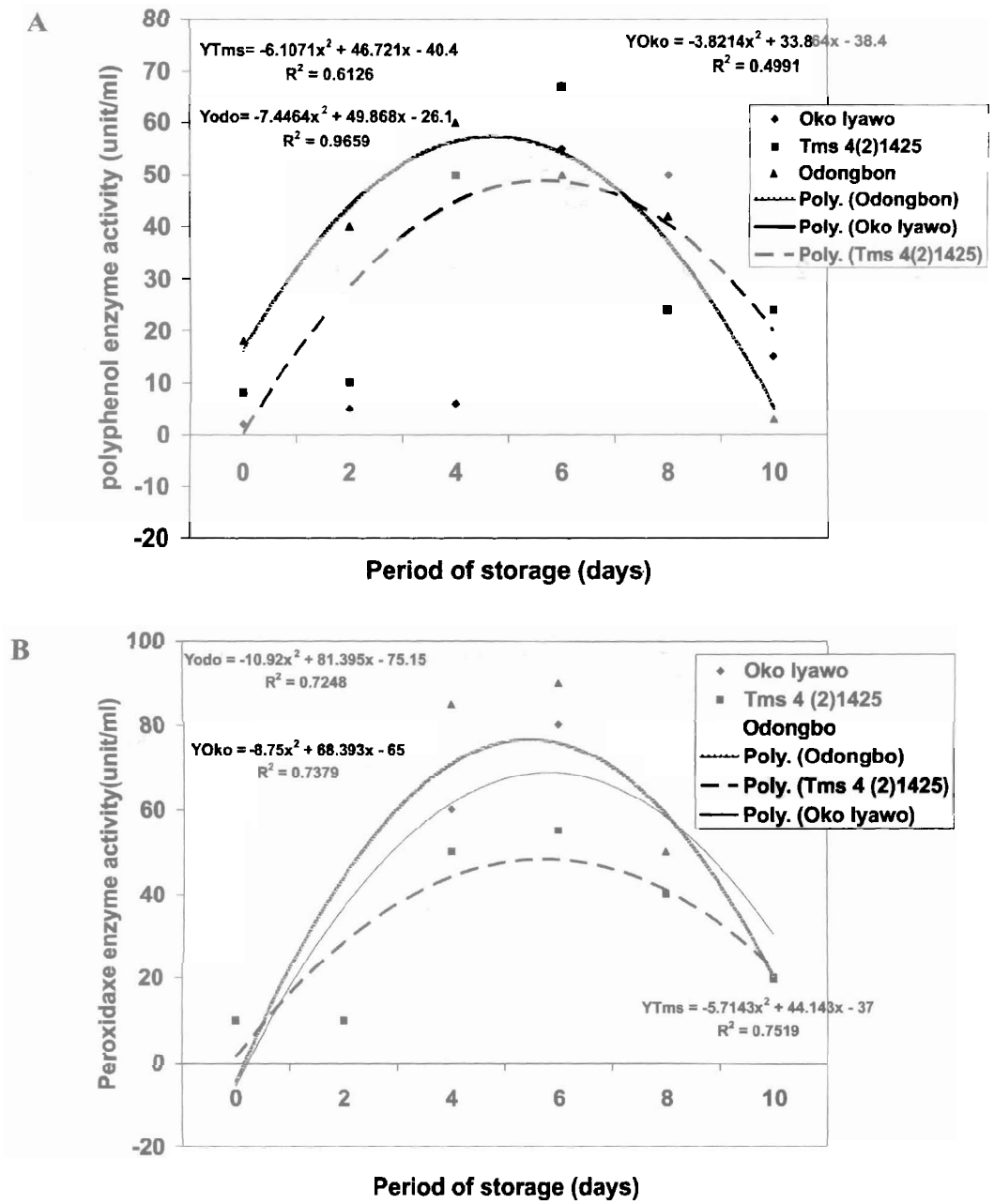



**Fig. 1: Changes in oxidative enzymes activities of cassava tubers during storage.**

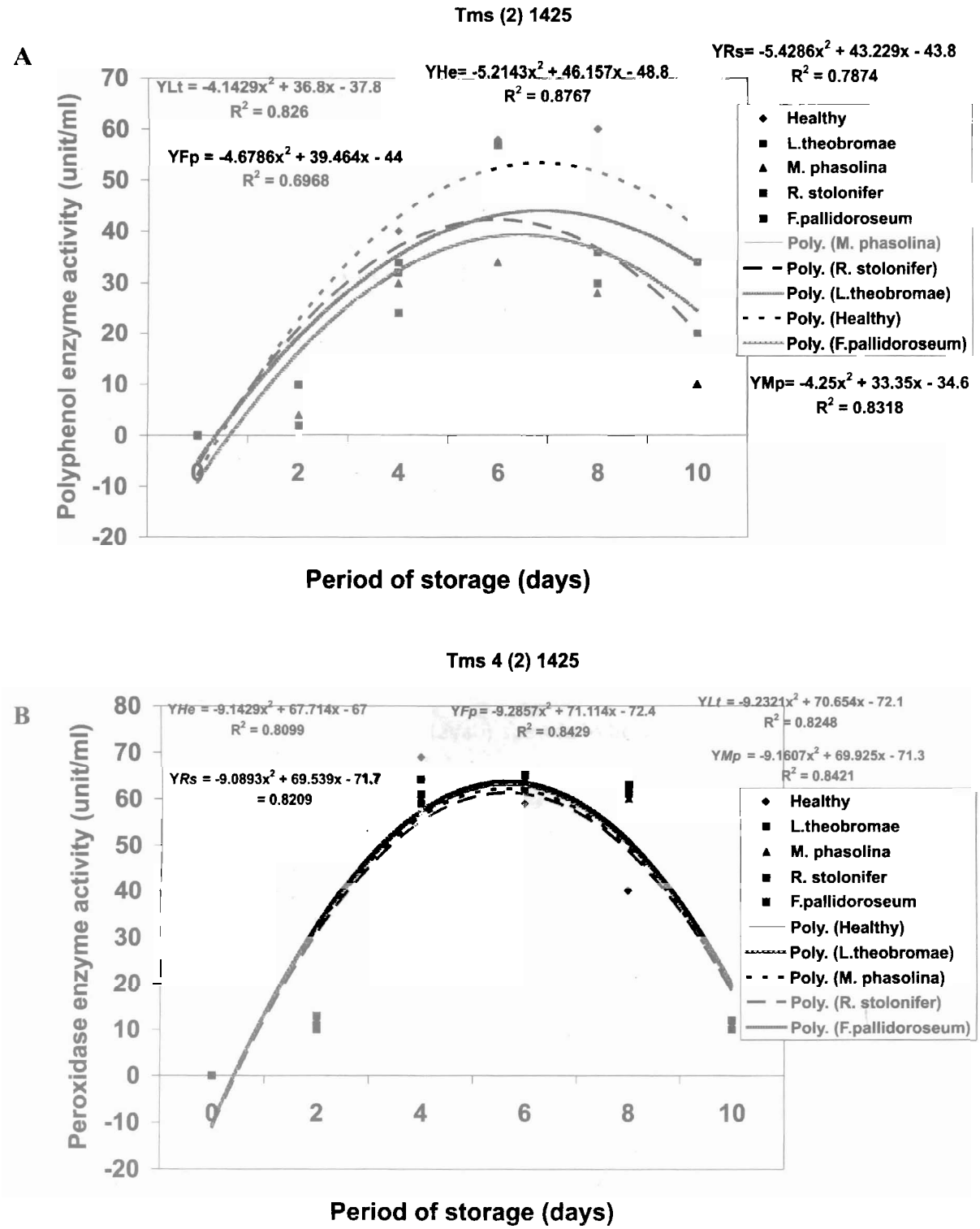



**Fig.2: Activities of polyphenol and peroxidase enzymes in the tuber of healthy and infected TMS (2)1425 variety of cassava during storage.**

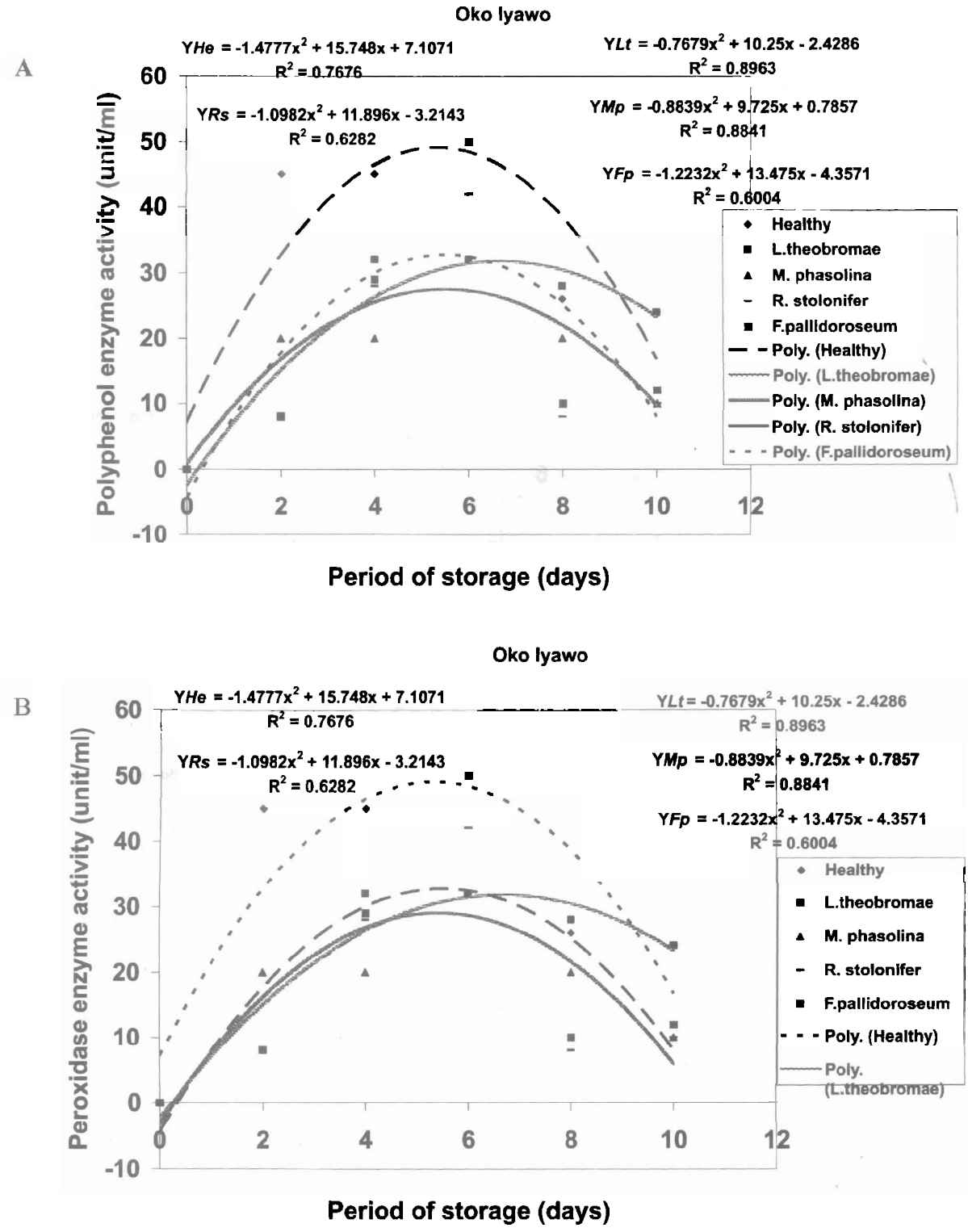



**Fig. 3: Polyphenol and peroxidase enzyme activity in the tuber of healthy and infected oko iyawo variety of cassava during storage .**

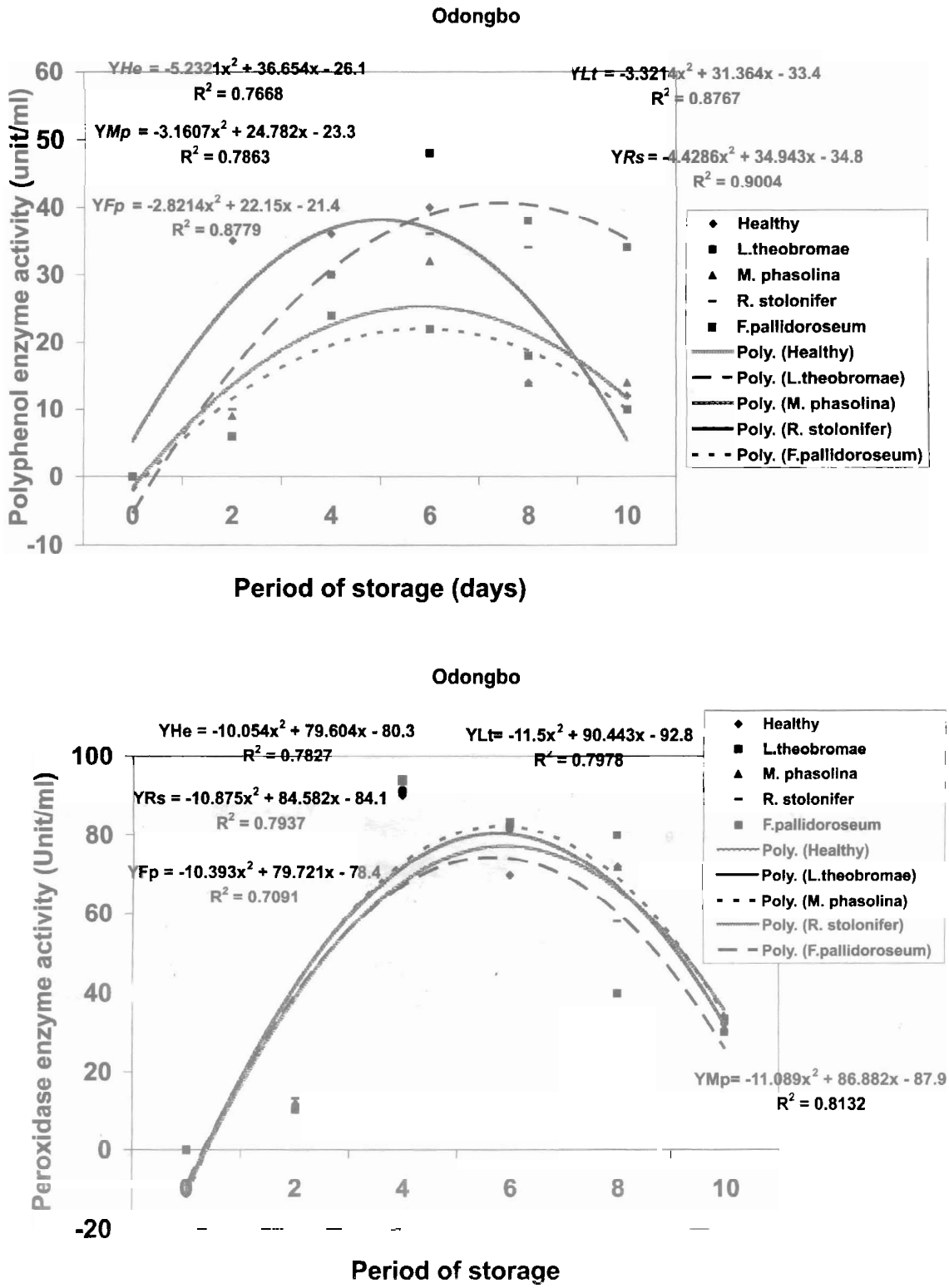
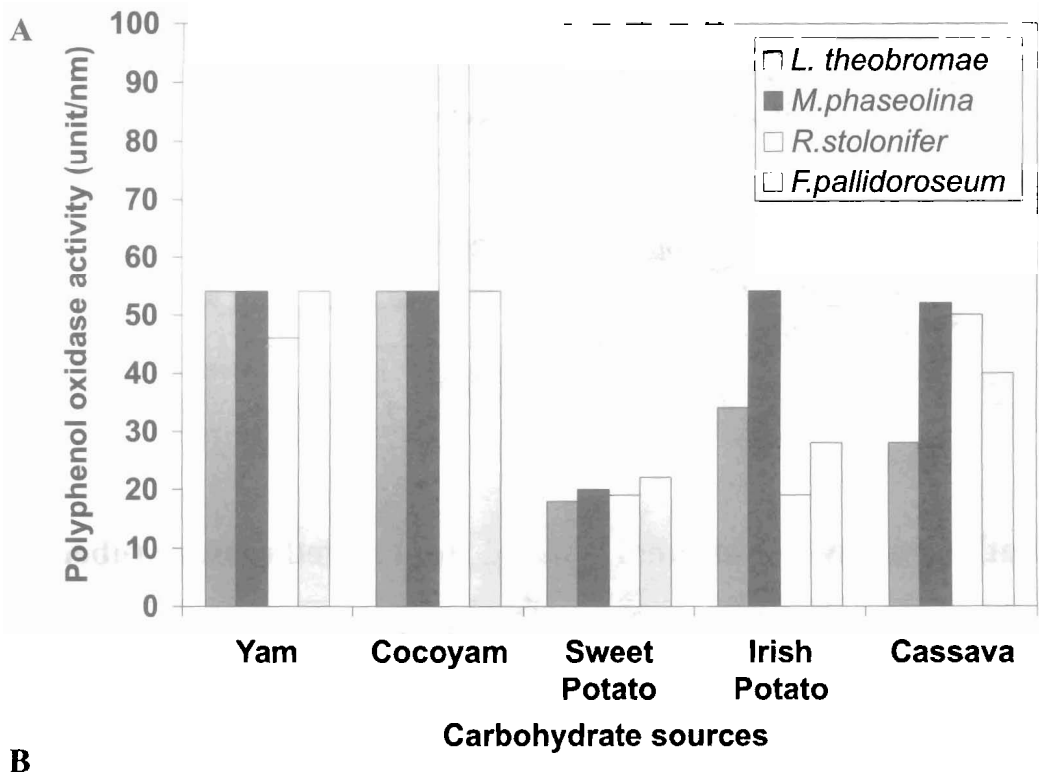



**Fig. 4: Polyphenol and peroxidase enzyme activity in the tuber of healthy and infected odongbo variety of cassava during storage.**



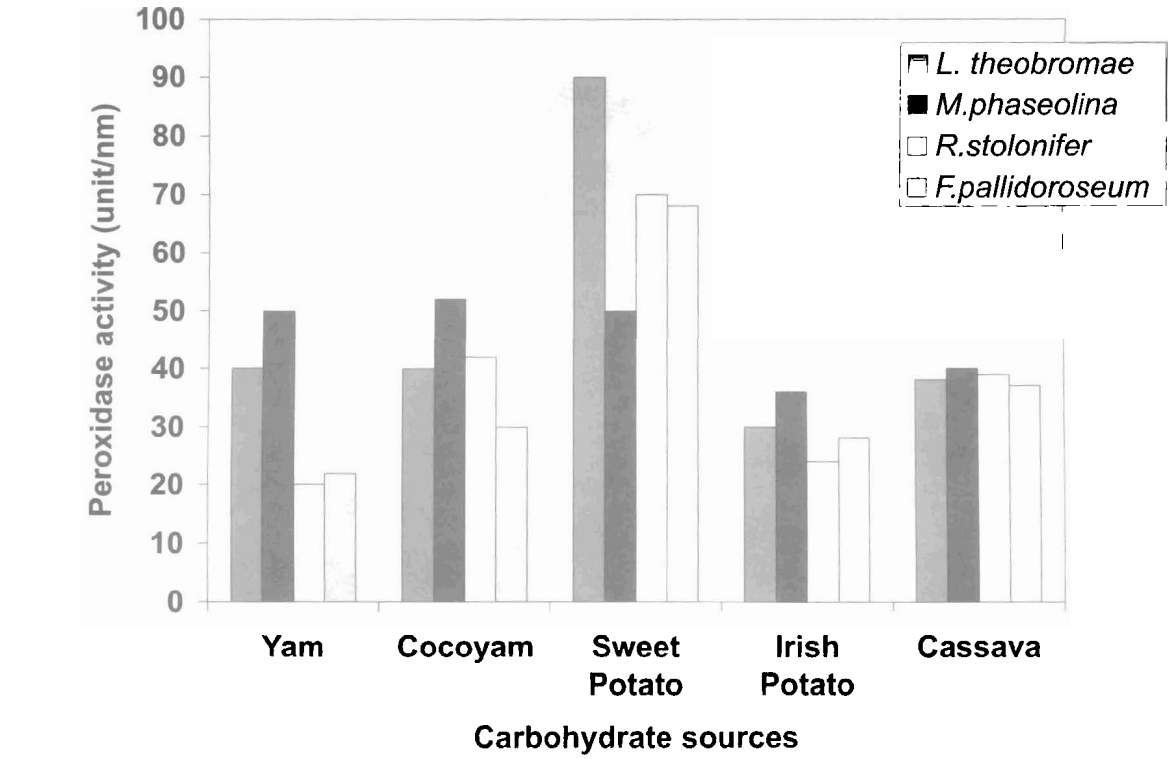




**Fig. 5: Mycelial dry weight of test pathogens showing the activities of the oxidative enzymes in the utilization of different carbohydrate sources.**

**Table 1: Pathogenic Indices of fungi associated with tuber rots of cassava**

| Fungi | Pathogenic Indices (%) |
|---|---|
| *Lasiodiplodia thoebromae* | 90 |
| *Macrophomina phaseolina* | 88 |
| *Rhizopus stolonifer* | 82 |
| *Fusarium pallidoroseum* | 76 |

**Table 2: Pathogenicity test of fungi isolated from rotted cassava tuber**

| Fungi | Indices of % rot | Remarks |
|---|---|---|
| *Lasiodiplodia thoebromae* | **3%** | Exudation of pungent smelling liquid |
| *Macrophomina phaseolina* | **3%** | Exudation of sweet smelling liquid |
| *Rhizopus stolonifer* | **2%** | Dry rot |
| *Fusarium pallidoroseum* | **2%** | Dry rot |