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Review

Review of challenges in the escalation of metal-biosorbing processes for wastewater treatment: Applied and commercialized technologies

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Despite the eminent consequences, wastewaters containing toxic heavy metals are still discharged in the environment without prior treatment, certainly because of the high cost associated with the effective management of these effluents. Difficulties emanated from the fact that most of the technologies available for the remediation of the pollution require high capital and operational cost, which cannot be justified for the treatment of diluted effluents. Biosorption technique for its relatively competitive cost has been immensely investigated in laboratories for remediation of heavy metals polluted effluents. This paper reviews the major progresses achieved in the field of biosorption technology since it was first introduced; enhancement of the performance of suitable biosorbents through regeneration and immobilization techniques are some of the approaches that have contributed to improve metal removal processes. Despite some few progresses, efforts devoted in the development of biosorption technology have not been translated into successful implementation everywhere, hence, the persistence of problems related to pollution of water sources by toxic heavy metals in most part of the world. Challenges hindering the commercialization of biosorption technology are clearly discussed and critical aspects related to the characteristics of waste effluents and potential of biosorbents are highlighted. Suggestions are made for consideration of hybrid technologies, carefully designed and informed by the complexity of waste effluents.

Key words: Mine waste, toxic heavy metals, biosorption technology, immobilization, desorption, hybridization of techniques, escalation of processes.

INTRODUCTION

The dispersion in the environment of toxic heavy metals mostly from hydrometallurgical plants has led to deterioration of the quality of water sources (Figure 1) all over the world. The presence of toxic heavy metals in ground and surface waters represent a serious threat to human health, especially in developing countries where communities in rural and poor urban areas rely on untreated water for their basic needs.

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Conventional methods or physic-chemical methods such as ion exchange, activated carbon, and electrodialysis used for removal of metals from solutions have been effective in some applications. However metal pollution of environmental waters could not be effectively remediated with such techniques because of the relatively low level of metals in such solutions. Hence, researches carried out over the years have mainly focused on ecological and cheap approaches to resolve the problem. A host of biosorbents have so far been tested for their potential in metal uptake with various successes (Volesky and Holan, 1995; Zhou et al., 1998; Matsunaga et al., 1999; Murphy et al., 2007; Vijayaraghavan and Yun, 2008). The extensive and desperate search for adequate biosorbents has led researchers to investigate the potential of microorganisms. Microorganisms have a large surface area, quick growth and can be obtained at relatively low-cost from their biotope or as industrial wastes (Park et al., 2010). Their cell surface contains identified active groups mainly responsible for passive/chemical uptake of metal from their environment. They can also sequester metal into the cytoplasm by active mechanism involving specific proteins (Fosso-Kankeu et al., 2011). Bacteria, fungi, algae and seaweeds are among the microorganisms often used in biosorption processes; although exhibiting different affinities for metals, microbial biosorbents have shown better adsorption capacities than other sorbents in most cases (Vieira and Volesky, 2000). However, most of experiments conducted at bench scale have never been escalated to industrial level, for various reasons. First of all, the use of living cells has mainly suffer the drawback of cell inhibition and cost of media from production; on the other hand, some dead biomasses collected from the environment have lower adsorption capacities (Wang and Chen, 2006; Volesky, 2001) and pretreatment required to obtain suitable biosorbents with high adsorption capacity will enhance the process cost. The quest for better microbial sorbent over the years has motivated consideration of new technical approaches which are intended for the improvement of the adsorption potential of microbial sorbents and increase of their life span. For example the idea of immobilization of cells was suggested by many researchers for minimization of cells degradation and facilitation of solid/liquid separation (Vijayaraghavan and Yun, 2008; Wang and Chen, 2009); while, the regeneration and reuse of cells has been considered in biosorption processes for the reduction of the cost of biomass production. Despite significant improvement/optimization of biosorption processes at bench scale, numerous attempts to escalate the process at industrial level have been unsuccessful.

This review extensively covers current challenges in the application of biosorption technology at large scale, and then substantiates the different scientific approaches which have been contemplated for improvement of biosorption processes. Reasons for failure in the escalation of biosorption processes are discussed and remedies suggested based on successful cases of commercialization of the technology.

THE POTENTIAL OF MICROORGANISMS FOR METAL UPTAKE

Prior to its uptake, metal ions firstly come into contact with the cell membrane of microorganism. It is reported (Kefala et al., 1999) that in the first five to fourty minutes of exposure, the passive or physic-chemical mechanism is mainly responsible of the removal of metal ions from solution. This mechanism occurs through interaction of metal ions with the functional groups present on the cell surface. Although extracellular polysaccharides of all bacteria are involved in metal binding (McLean et al., 1992), the main components responsible for metal-binding capacity of the cell wall in Gram-positive bacteria are anionic functional groups present in the peptidoglycan, teichoic acids and teichuronic acids, while in Gram-negative bacteria, peptidoglycan, phospholipids and lipopolysaccharides play the major role (Vijayaraghavan and Yun, 2008).

The cell walls of fungi and algae are different in chemical composition from prokaryotic cell walls. The cell walls of fungi are dominated by polysaccharide completed with proteins, lipids, polyphosphates and inorganic ions. Algal cell walls are similar to the one of fungi in structure and contain xylans, pectin, mannans, alginic acids or fucinic acid.

The functional groups which are directly responsible for the binding of metals at the surface of all the microorganisms have been listed by Talaro and Talaro (2002) and include: hydroxyl, carboxyl, amino, ester, sulfhydryl, carbonyl terminal end, carbonyl internal and phosphate. These groups mainly influence cell surface affinity for ligands as illustrated in the Table 1.

Metal binding to cell wall occurs through two basic mechanisms: stoichemistry interaction between the metal and the reactive chemical groups (phosphate, carboxyl, amine, phosphodiester etc) in the cell wall, and inorganic deposition of metals (Gupta et al., 2000). The passive adsorption of metal species by various functional groups on microbial cell wall includes non-metabolic mechanisms such as ion exchange, complexation, chelation, coordination, microprecipitation and reduction (Volesky, 1990a, b; Liu et al., 2002).

As discussed above, microorganisms have physiological characteristics suitable for the removal of metal from solutions; however the performance of microbial sorbents may vary depending on the strain used and the metal in solution. This is mainly due to the fact that microorganisms may have different active groups on their cell wall and the extracellular polymeric substances (EPS) produced or excreted by some of them are often not identical (Wang and Chen, 2006). This may explain why some microbial sorbents outperform others; Genre of *Bacillus*

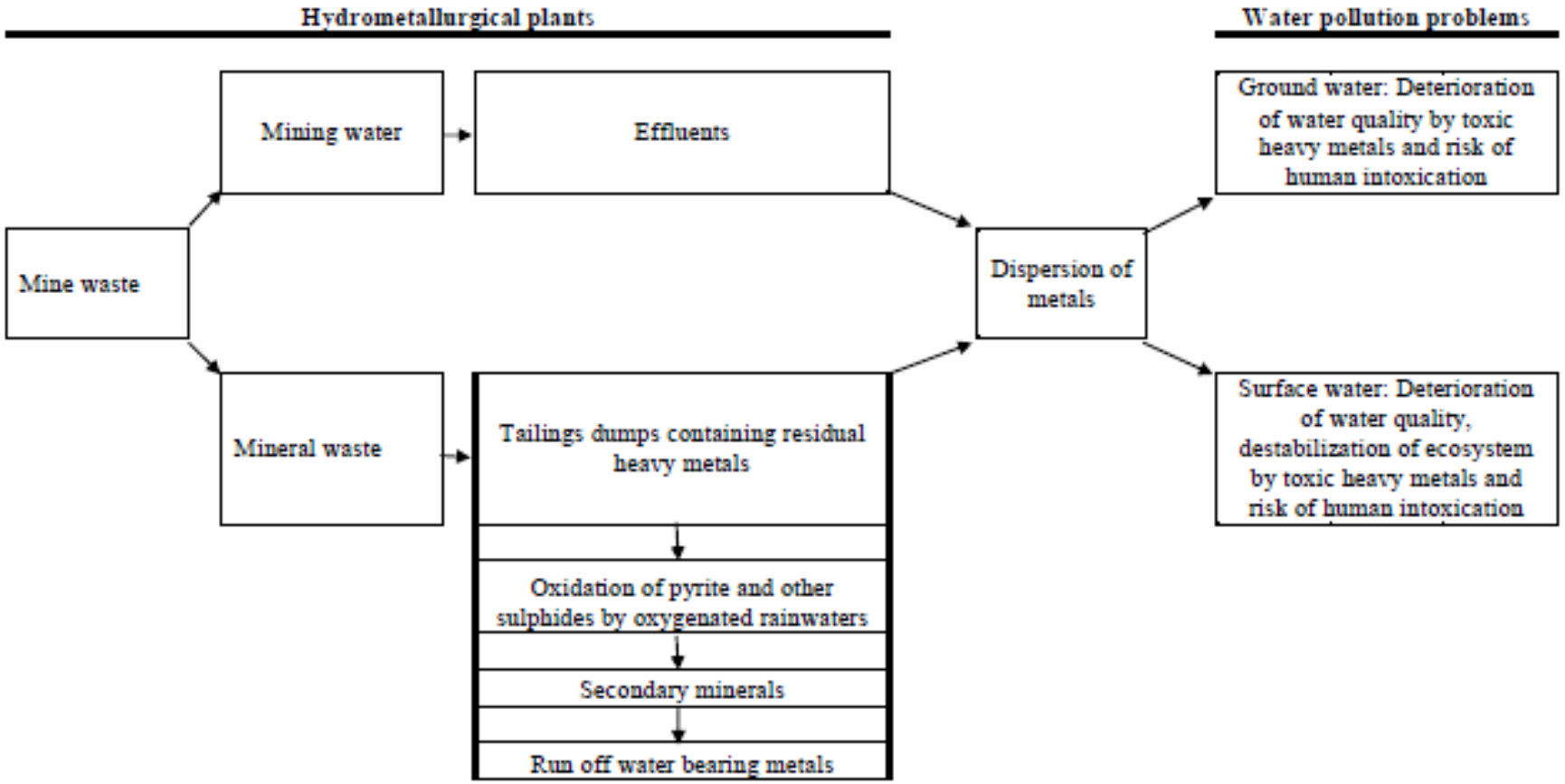

Figure 1. Dispersion of toxic heavy metals from mine wastes

Table 1. The ligands present in biological system and three classes of metals (Remacle, 1990).

Ligand class	Ligand	Metal class
Ligands preferred to Class A	F^-, O^{2-}, OH^-, H2O, CO_3^{2-}, SO_4^-, $ROSO_3^-$, NO_3^-, HPO_4^{2-}, ROH, $RCOO^-$, C=O, ROR	Class A: Li, Be, Na, Mg, K, Ca, Sc, Rb, Sr, Y, Cs, Ba, La, Fr, Ra, Ac, Al, Lanthanides, Actinides
Other important ligands	Cl^-, Br^-, N_3^-, NO_2^-, SO_3^{2-}, NH_3, N_2, RNH_2, R_2NH, R_3N, =N-, -CO-N-, R, O_2, O_2^-, O_2^{2-}	Borderline ions: Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, Cd, In, Sn, Sb, As
Ligands preferred to Class B	H^-, I^-, R^-, CN^-, CO, S^{2-}, RS^-, R_2S, R_3AS	Class B: Rh, Pd, Ag, Lr, Pt, Au, Hg, Tl, Pb, Bi

and *Pseudomonas* among bacteria, *Aspergillus*, *Rhizopus* and *Penicillium* among fungi have been identified as good biosorbents (Bai and Abraham, 2003; Tan and Cheng, 2003; Park et al., 2005; Binupriya et al., 2006; Tunali et al., 2006; Uslu and Tanyol, 2006; Vijayaraghavan and Yun, 2008). As far as selectivity of metal is concerned, the physio-chemical nature of metal and active groups on cell surface makes biosorption selective. The affinity or the tendency of microorganisms to preferably bind a given metal is influenced by factors such as the ionic radius and the electronegativity (Mattuschka and Straube, 1993; Brady and Tobin, 1995; Chong and Volesky, 1995).

The polarizing power of a cation increases as the ionic radius decreases, implying that lower ionic radius may lead to reduce biosorption; on the other hand, the electronegativity which represents the ability of an atom to attract electron for the formation of ionic bond, increases with the affinity of microbial biomass for a metal (Allen and Brown, 1995; Brady and Tobin, 1995; Gabr et al., 2008). Mattushka and Straube (1993) observed that biosorption was selective when attempting to remove metals using *Streptomyces* waste biomass. The equilibrium constant of the Langmuir isotherm was reported (Gabr et al., 2008; Baysal et al., 2009) to positively correlate to the strength of the binding sites on the biomass surface and has been used to illustrate the sorption affinity of microbial biosorbents for metal ions. Gabr et al. (2008) established a positive correlation between an increase of the equilibrium constant with the sorption affinity of *P. aeruginosa* ASU 6a for lead, while on a similar basis Fosso-Kankeu et al. (2011) showed selective binding of heavy metal ions (Ni^{2+} and Co^{2+}) on Bacillaceae bacterium in the presence of light metal ions (Ca^{2+} and Mg^{2+}). Sorption of metal on EPS produced by some microorganisms was also found to be selective; Greater selectivity of copper over cobalt was observed by Jang et al. (1995) when performing competitive uptake on Na-alginate extracted from *M. pyrifera* or *L. hyperborea*. This selectivity was attributed to the guluronic content, as also confirmed by the works of Figueira et al. (1997) and, Haug and Smidsrod (1965; 1967).

Intracellular uptake of metals or bioaccumulation is used by living cells for the uptake of metal from the environment and differs in biosorption mechanism. It can occur through two uptake systems; a fast, unspecific and constitutively expressed system which is driven by the chemiosmotic gradient across the cytoplasmic membrane of bacteria; the second system is much more slower, specific and is a metabolic process that requires hydrolysis of adenosine triphosphate (ATP) as source of energy. Metals Sequestered from the environment interact and bind to specific proteins or chelatins and are transported into the vacuoles and other intracellular sites. A number of protein families are involved in the transport of metals into the cytoplasm of microorganisms; these proteins are specific and are not found in all bacteria. However, they can be engineered in foreign strains, especially to improve their tolerance for toxic heavy metals. The *cnr* operon which is originally present in *Cupriavidus metallidurans* and mediates resistance to nickel and cobalt was recently expressed by Fosso-Kankeu et al. (2012) in foreign Gram-positive strain namely Bacillaceae bacterium to improve their capacity to uptake nickel from solution. This innovative approach of expressing the Resistance-Nodulation-Cell division in Gram-positive strain was inconclusively suspected to be the result of a mutation.

Bioaccumulation is a slower process as compared to biosorption (Malik, 2004; Vijayaraghavan and Yun, 2008). In growing/living cells, metal uptake is a biphasic process, starting with the rapid phase of biosorption, followed by a slower phase of bioaccumulation. There is a conflicting opinion about which of the living or dead cells are better biosorbents for remediation processes. Some researchers (Vijayaraghavan and Yun, 2008) argued that biosorption has a higher degree of uptake, while other findings (Matsunaga et al., 1999; Perez-Rama et al., 2002; Malik, 2004) suggest that intracellular accumulation of metals such Cd by growing culture of marine microalgae accounts for most of the uptake.

MICROBIAL SORBENTS AS LIMITING FACTORS IN BIOSORPTION PROCESSES

The increasing volume of polluted effluents discharged in the environment by industries requires appropriate measures to effectively address the problem. The limitations of the conventional techniques with regards to the treatment of large volume of diluted effluents could not be over emphasized; better technologies are too costly to be used for daily running of bioremediation plants. In addition, chemical treatment often results in the production of toxic sludge and waste by-products. All these disadvantages have led to exploration of alternative method namely biological treatment for removal of toxic metal from effluents. This method presents a number of advantages such as low cost (biosorbents are abundant materials easily produced or collected as waste biomass from industries), high adsorption capacity of biosorbents, possible regeneration of biosorbents and recovery of metals and no sludge produced during the process. This has positioned biosorption as one of the most attractive Techniques for remediation of polluted effluents. However, several attempts to implement laboratories' concepts at large scale processes did not meet the predicted expectations for reasons still speculated and under investigation. Few of those reasons will be discussed in this review followed by palliative measures undertaken for substantial improvement of the process.

It has been observed that the use of living cells requires extra cost for their production, making the process relatively costly. On the other hand, living cells are sus-

ceptible to harsh environmental conditions such as extreme pH and temperatures as well as relatively high concentration of metals; therefore the use of living biomasses for metal uptake can be affected by the inhibition of cells (Eccles, 1995). During investigation, Tangaromsuk et al. (2002) observed growth inhibition of *Sphingomonas paucimobilis* biomass exposed to 25 to 200 mg/L of cadmium. Fosso-Kankeu et al. (2012) reported reduction of bacteria surface area following inhibition of living cells by relatively high concentration of nickel; this affected the adsorption capacity and the prediction of microbial sorbent behaviour.

Studying the bioaccumulation of copper (II) and nickel (II) by the non-adapted and adapted growing *Candida* sp., Donmez and Aksu (2001) made and important finding; the adsorption capacity of both cells decreased with increase in initial concentration of metals; while the copper's uptake capacity of adapted *Candida* sp. (36.9 mg/g dry weight) was greater than the one of non-adapted *Candida* sp. (23.1 mg/g dry weight) at relatively higher initial concentrations of copper.

Although adequate for prediction of adsorption performance at the bench scale, the use of suspended biomasses is unsuitable for practical application, because of: 1) low mechanical strength: harsh conditions are likely to degrade microbial sorbents and therefore affect the stability and chemical structure of active groups on cell surface; 2) easy cell loss: free cells are difficult to control and are likely to contaminate the treated water, this may come up with solid-liquid separation problems; 3) ineffective recovery of metals: It will be difficult to recover all the metals adsorbed at the surface of the biomass, since loaded Biomasses are dispersed in the solution; and 4) Inability to regenerate/reuse biosorbents: as the biosorbents are degraded under extreme conditions, their adsorption capacity will be considerably reduced.

One of the predicted advantages of biosorption was the lower cost of the process mainly due to the possibility of using waste biomass from the environment. However it has been noticed that some of the waste biomasses are often supplied wet and there is a need of drying the raw biomasses to prevent its degradation during storage (Volesky, 2007). Furthermore, some of the waste biomasses such as *Saccharomyces cerevisiae* from fermentation industries, have low adsorption capacity because of the transformation undergone in the industries and are therefore considered as mediocre biosorbents in comparison to other biomasses (Volesky, 1994; Bakkaloglu et al., 1998; Wang and Chen, 2006).

DEVELOPMENTS IN BIOSORPTION TECHNOLOGY

After various attempts of biosorption experiments using hundreds of biosorbents for adsorption of metals under different Physic-chemical conditions, it has emerged that optimum conditions observed at bench scale are not sufficient to achieve the objectives specified for the treatment of wastewater in the bioremediation plants. To address some of the limitations elucidated above, researchers have explored methods to stabilize biosorbents, minimise their production costs and increase the chance of recovery of valuable metals.

Immobilization

For the reasons already discussed, free cells are not adequate for application of biosorption processes; it is therefore important to attach microbial sorbent to suitable matrix prior to use in conventional unit systems, such as packed/fluidized bed reactors and continuous stirred tank reactors; therefore ensuring optimum uptake capacity and reuse over several cycles.

There are a number of techniques used for immobilization (Table 2) of microbial biosorbent and they vary according to the physical mechanism exploited; these techniques include: entrapment within a porous matrix, cross-linking induced by chemical agents; encapsulation which is similar to entrapment, but the microbial sorbents are free-floating within a capsule-like membrane walls; and attachment or adsorption on inert carriers. The latter involves weak forces such as van der Waals forces, ionic and hydrophobic interactions and hydrogen bonds which are responsible for the formation of bonds (Flickinger and Drew, 1999; Guisan, 2006; Kumar, 2009).

The varied chemistry and the nature of biomass as well as the polymeric matrices required that sufficient care is taken during the choice of the immobilization matrix to carry out biosorption processes at industrial scale. Immobilization techniques have been used for enhancement of microbial sorbents for removal of different metal ions from synthetic solutions or real environmental wastewaters. A number of polymeric matrices including poly (vinyl formal), polysulfone, polyurethane, alginate, polyacrylamide, k-carrageenan and polyethylenimine (PEI) were used in the laboratory to immobilize Rhizopus oryzae in an attempt to improve biosorption of copper (Al-Hakawati and Banks, 2000). Developed porous polysulfone beads containing immobilized nonliving biomasses were used by the US Bureau of Mines, Salt Lake City Research Center for extraction of metal contaminants from wastewaters (Jeffers et al., 1991; Jeffers and Corwin, 1993; Beolchini et al., 2003). One of the main challenges often encountered following immobilization of microbial sorbents is the mass transfer resistance which slow the attainment of equilibrium; a delay in the attainment of equilibrium was observed by Vijayaraghavan et al. (2007) during the uptake of dye using *C. glutamicum* immobilized within a polysulfone matrix. Several works have reported improvement of the adsorption capacity of biosorbents after immobilization on matrices (Table 3), however the cost of immobilization together with other disadvantages (Table 2) have to be carefully considered

Table 2. Available techniques for the immobilization of biomass.

Technique	Examples of matrices/reagent	Advantage	Disadvantage	Reference
Entrapment	Polysulfone, alginate, polyurethane, polyacrylamide, etc	Known cheap	Mass transfer resistance	Gorecka and Jastrzebska, 2011; Park et al., 2010; Volesky, 2001; Gilson and Thomas, 1995; Jeffers et al., 1993; Trujillo et al., 1991
Cross-linking	Nitroacetic acid, epoxides, ethylene glycol diglycidiyl ether (EGDE), glutaraldehyde, divinylsulfone, formaldehyde, etc	Increased strength	Loss of activity, not universal	Gorecka and Jastrzebska, 2011; Park et al., 2010; Volesky, 2001; Leusch et al., 1995
Encapsulation	Alginate, chitosan, maltodextrin, cellulose, etc. Often coated with chitosan, polyvinyl acetate (PVA), gelatin, etc	Prevent biosorbents leakage, higher catalyst densities	Mass transfer resistance, fragile capsules	Gorecka and Jastrzebska, 2011; Park et al., 2010; Volesky, 2001; Chang, 1992; Chang, 1995
Adsorption	Active charcoal, ceramic, glass bead, sand, carbon nanotubes (CNTs), etc	Higher biomass loading, simple and cheap technique	Possible leakage of biosorbents, unstable binding	Gorecka and Jastrzebska, 2011; Park et al., 2010

and addressed for successful application of biosorption at industrial level. Polysulfone beads were identified by few researchers (Jeffers et al., 1991; Veglio et al., 1998; 1999) as suitable immobilization matrix because of their lower cost and mechanical strength. Recently, some researchers attempted to use carbon nanotubes (CNTs) as immobilizing support (Liu et al., 2009). CNTs are divided into single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs), which are considered as good candidates adsorbers because of their hollow and layered nanosized structures (Chen et al., 2009; Liu et al., 2009; Ren et al., 2011).

Regeneration and reuse of biosorbents

Among the available and realistic ways of minimising the cost in the application of biosorption process and still achieving the maximum removal of toxic metals from wastewater, regeneration and reuse of biosorbents are of utmost importance as they provide the opportunity not only to cut down the cost of biosorbents, but also to recover valuable and scarce metals. It is however equally important to select good quality eluent, which is less aggressive to the biomass, effective and cheap. Different categories of reagents susceptible to serve as eluting agents are available on the market and include mineral acids (HCl, H_2SO_4, HNO_3, H_3PO_4, etc), alkalis (NaOH, NH_4OH, etc.), organic solvents (ethanol, methanol, acetone, etc) and others (EDTA, Na_2CO_3, $CaCl_2$, $KHCO_3$, KSCN, etc.). These reagents all have the potential to unbind the metal from the biosorbents, but the mechanism of desorption may vary; three basic mechanisms of desorption have been identified by some authors (Kapoor et al., 1999; Lezcano et al., 2011): Formation of insoluble compound with eluent can lead to precipitation of immobilized metal (example of eluent: H_2SO_4); Competition among ions for the binding sites on loaded biomass may lead to the displacement of the immobilized ion by the ion in solution, it is an ion exchange mechanism (example of eluents: mineral acids, alkalis, etc.); Sharing of electron between immobilized metal and eluent often results in complexation (example of eluents: EDTA, Na_2CO_3, etc.).

For desorption of cation, mineral acids are often preferred as they can quickly wash off the metals from the loaded biomass and produce metal rich effluent suitable for economic exploitation (Volesky, 2007; Lezcano et al., 2011). The main problem with mineral acids is the aggressiveness toward the biomass which can diminish the capa-city of the biosorbent in the next cycle. Desorbing heavy metals from loaded biomass of *Sargassum* seaweed, Davis et al. (2000) observed that was-hing with solutions of HCl rendered the biomass more fragile. Although large amount of immobi-lized metals can be easily desorbed with large volume of eluent, rapid elution, using the smallest quantity of eluent possible is desirable, in order to achieve high concentration of metals in minimum volume of effluent. Hence the need for the researcher to optimize the elution process through a trial experiment that will enable the adequate solid/liquid ratio (solid representing the loaded sorbent and the eluent being the liquid). Desorbing loaded biomass of *Sagassum* seaweed with calcium

Table 3. Performances of some polymers as immobilizing matrices of biosorbents for metal uptake.

Biosorbent	State of biosorbent	Biosorption technique	Matrix	Best matrix	Immobilization techniques	Maximum adsorption capacity first cycle	References
Aspergillus niger	Dead cells, powder form	Column	Polysulfone	Polysulfone	Adsorption	Cd(3.6 mg/g), Cu(2.89 mg/g), Pb(10.05 mg/g), Ni(1.08 mg/g)	Kapoor and Viraraghavan (1998)
Sargassum duplicatum	Dead cells, powder form	Column	Silica gel	Silica gel	Adsorption	Cu(280.1 µmol/g), Cd(130.5 µmol/g), Pb(113.7 µmol/g)	Suharso et al. (2010)
Ulva lactuca	Dead cells, powder form	Column	Agar	Agar	Adsorption	Cu(0.85 mmol/g), Zn(0.35 mmol/g), Cd(0.41 mmol/g), Pb(1.55 mmol/g)	Areco et al. (2012)
Phormidium laminosum	Dead cells, powder form	Batch	Polysulfone, epoxy resin	Polysulfone	Adsorption	Cu(19.83 mg/g), Fe(17.83 mg/g), Ni(16.1 mg/g), Zn(18.05 mg/g)	Blanco et al. (1999)
Pseudomonas fluorescens, *Microbacterium oxydans* and *Cupriavidus* sp	Living cells	Batch	Hydroxyapatite	Hydroxyapatite	Adsorption	Zn(0.433 mmol/g), Cd(0.09 mmol/g)	Piccirillo et al. (2013)
Phaseolus vulgaris L.	Plant, powder form	Column	Silica gel	Silica gel	Entrapment	Ni(0.0017 mol/g)	Akar et al. (2009)
Bacillus strain CR-7	Dead cells, powder form	Batch	Sodium alginate, gelatin, polyvinyl alcohol	Sodium alginate (2%)		Cu(19 mg/g)	Xu et al. (2011)
Cupriavidus, *Sphingobacterium*, *Alcaligenes*	Living cells	Column	Alginate, pectate and a synthetic cross-linked polymer	Synthetic cross-linked polymer	Entrapment	Zn(1.7 mM/g), Cd(0.9 mM/g)	Pires et al. (2011)
Mentha arvensis	Living cells	Batch	Sodium alginate (2%)	Sodium alginate (2%)	Entrapment	Cu(104.48 mg/g), Zn(107.75 mg/g)	Hanif et al. (2009)
Phanerochaete chrysosporium	Living cells	Batch	Iron oxide magnetic nanoparticles (MNPs) and Ca–alginate	Iron oxide magnetic nanoparticles (MNPs) and Ca–alginate	Entrapment	Pb(185.25 mg/g)	Xu et al. (2013)
Rhizopus nigricans	Dead cells, powder form	Batch	Calcium alginate, polyvinyl alcohol (PVA), polyacrylamide, polyisoprene, and polysulfone	Polysulfone	Entrapment	Cr(VI) 119.2 mg/g	Bai and Abraham (2003)

Table 3. Contd.

Trichoderma viride	Dead cells, powder form	Column	Ca-alginate	Ca-alginate	Adsorption	Cr(6.9 mg/g), Ni(6.9 mg/g), Zn(4.95 mg/g)	Kumar et al. (2011)
Chlorella sp. and *Chlamydomonas* sp.	Living cells	Batch	Sodium alginate	Sodium alginate	Entrapment	Cu(33.4 mg/g), Zn(28.5 mg/g)	Maznah et al. (2012)
Sargassum baccularia	Dead cells, powder form	Batch	Polyvinyl alcohol	Polyvinyl alcohol	Adsorption	Cu(5 mg/g)	Hashim et al. (2000)

salt, Davis et al. (2000) observed a decrease from 95% to less than 50% elution efficiency after increase of solid/liquid ratio.

Desorption has been performed on suspended as well as immobilized cells. It is quite easy to elute metal from suspended cells than from immobilized cells (Atkinson et al., 1998); however suspended cells are more susceptible to degradation and therefore reduction of adsorption capacity. Using 1% (w/v) $CaCl_2$/HCl-solution at pH 3 to desorb suspended-loaded biomass of *Sargassum*, Volesky et al. (2003) achieved a desorption efficiency around 95% for a maximum of seven cycles, but the biomass loss was 21.6%. In a separate study, Bai and Abraham (2003) investigated chromium (VI) adsorption-desorption on immobilized fungal biomass; using the best matrix (polysulfone), they observed that the biomass beads could be regenerated and reused in more than 25 cycles and the regeneration efficiency was 75 to 78%.

The physiological state of microbial sorbent (dead or leaving cells) and the affinity of the biosorbent for the metal are parameters that can affect the effectiveness of regeneration and reuse of biosorbent (Table 4).

Although total desorption of sorbates attached to biosorbent through passive (metabolic independent) mechanism are easily achieved, it is not the case when the sorbates are inside the cell (bioaccumulation in living cells). Total recovery of intracellular bound metals is possible only if the cells are destroyed using techniques such as incineration or dissolution into strong acids or alkalis (Park et al., 2010).

It is known that ineffective desorption of biosorbent (that may result from high affinity of sorbate or weakness of eluent) diminishes the adsorption capacity in the next cycle as residual ions on biosorbent's surface occupy the binding sites and will compete with ions in solution. On the other hand, if the eluent is too strong, it will result in low displacement of desorbing ions during the next cycle. The need to evaluate the performance of different eluents at various dose prior to selection, should therefore be emphasized.

Hybridization of techniques

Improvement of biosorption processes through immobilization and/or regeneration of biosorbents has not yet contributed to the success expected. In regards of the outcomes of the number of attempts by researchers exploiting these avenues to substantiate the benefits of the unequivocal potential of biosorption, it could be said that alternative measures suggested by previous authors (Tsezos, 2001; Malik, 2004; Wang and Chen, 2006; 2009) and consisting of the use of hybrid technologies (intrabiotechnological or intertechnological) involving biosorption have to be seriously considered and thoroughly investigated. The heterogenic nature of environmental waste waters which are contaminated not only with toxic heavy metal ions, but also with considerable amount of light metal ions, organic matters and therefore higher total dissolved solids. All these can significantly interfere with the performance of the biosorbents and biosorption system; Light metal ions will compete with the ions of interest for the binding sites while the organic matters and other solids in solution will contribute to rapid clogging of the column or saturation of biosorbent surface (Vijayaraghavan and Yun, 2008). It is therefore clear that any venture in the treatment of environmental waste waters relying on the unique application of biosorption will hardly succeed in achieving the effective cleanup of metal pollutants.

An approach based on the simultaneous or consecutive application of different techniques bearing in mind the affinity principle, could help address the above challenges in an integrated and effective manner. A number of techniques that can be combined with biosorption (Figure 2) have been suggested and include bioprocesses such as bioreduction and bioprecipitation with other processes namely electrochemical processes, chemical precipitation, flotation, membrane technology and more (Tsezos, 2001; Wang and Chen, 2006). Successful removal of inorganic and organic pollutants from environmental effluent was achieved by Diels et al. (2001) using a combination of metal biosorbing and bioprecipitating bacteria in moving bed sand filters (effective bed height, 2 m) biofilm. Another hybrid approach

Table 4. Examples of Eluants used for the recovery of metals and regeneration of biomasses.

Biomass	Eluants used	Best eluant(s)	Maximum sorption capacity (cycle 1)	Maximum % desorption (cycle 1)	Maximum Adsorption capacity (last cycle)	Maximum loss of biomass, total number of cycle	Reference
Cyanobacterial mats	HCl, $CaCl_2$, SDS, HNO_3, NaOH	0.1 mM HCl	Pb(1 mM/g), Cu(0.35 mM/g), Cd(0.3 mM/g)	Pb(93.4%), Cu(92.7%), Cd(84.6%)	Pb(1 mM/g), Cu(0.35 mM/g), Cd(0.3 mM/g)	11%, 6	Kumar and Gaur (2011)
Streptoverticilliu m cinnamoneum	HCl, HNO_3, EDTA, H_2SO_4, Na-citrate, $NaHCO_3$, Na_2CO_3, KCl	0.1 M HCl, 0.1 M HNO_3, 0.1 M EDTA	Pb(57.7 mg/g), Zn(21.3 mg.g)	Pb(10%), Zn(90%)	Pb(43 mg/g), Zn(14 mg/g)	24.3% (Na2CO3), 3	Puranik and Paknikar (1997)
Phormidium laminosum	0.1 M HCl	0.1 M HCl	Cu(950 µg), Fe(900 µg), Ni(750 µg), Zn(900 µg)	Cu(90%)), Fe(85%), Ni(95%), Zn(120%)	Cu(900 µg), Fe(950 µg), Ni(1000 µg), Zn(1000 µg)	(immobilized biomass), 10	Blanco et al. (1999)
Enterobacter sp. J1	0.1 M HCl	0.1 M HCl	Pb(50 mg/g), Cu(32.5 mg/g), Cd(46.2 mg/g)	Pb(90%), Cu(90%), Cd(100%)	Pb(75%), Cu(79%), Cd(90%)	Na, 4	Lu et al. (2006)
Pithophora oedogonia	HCl(0.1 M), HNO_3(0.1 M), NaOH(0.1 M), H_2SO_4(0.1 M), $CaCl_2$(0.1 M), Na_2CO_3(0.1 M), EDTA(0.1 M)	HCl(0.1 M), EDTA(0.1 M)	Cu(23 mg/g), Pb(52 mg/g)	Cu(92.3%), Pb(96.2%)	Cu(12 mg/g), Pb(35 mg/g)	15%, 5	Singh et al. (2008)
Rhizopus nigricans	0.01N of acids, salts, alkalies, deionized distilled water and buffers	0.01 N NaOH, 0.01 N Na_2CO_3, 0.01 N $NaHCO_3$	Cr(VI)44.34 mg/g	Cr(VI)90 to 95%	Cr(VI)15 mg/g	Immobilized biomass-polysulfone), 25	Bai and Abraham (2003)
Sargassum	Hydrochloric, nitric, oxalic and diglycolic acids; calcium nitrate and chloride, EDTA-disodium	0.3 M of HCl	La(0.23 mmol/g), Eu(0.24 mmol/g), Yb(0.24 mmol/g)	La(90%), Eu(100%), Yb(100%)	La(0.25 mmol/g), Eu(0.39 mmol/g), Yb(0.23 mmol/g)	30%, 4	Diniz and Volesky (2006)

reported was conducted in two steps involving the bioleaching of toxic metals (Cu, Ni, Mn) by acidophilic sulphur-oxidizing bacteria in the first step, and precipitation (80 to 98%) of the leached metals in the next step by the activity of sulphate-reducing bacteria in an anae-robic bioreactor

Table 4. Contd.

Saccharomyces cerevisiae	0.1 M of HCl, HNO3, H_2SO_4, CH_3COOH and EDTA	H_2SO_4 pH1	Cr(III) 3.75 mg/g	Cr(III) 52%	Cr(III) 2 mg/g	17.50%, 3	Ferraz et al. (2004)
Spirogyra neglecta	0.1 M HCl	0.1 M HCl	Cu(30.17 mg/g), Pb(49.11 mg/g)	Cu(88.8 %), Pb(97.5%)	Cu(21.24 mg/g), Pb(38.33 mg/g)	Na, 10	Singh et al. (2012)
Streptomyces zinciresistens	0.1 M HCl	0.1 M HCl	Zn(160 mg/g), Cd(65 mg/g)	Zn(87.33%), Cd(98.11%)	na	Na, 1	Lin et al. (2012)
Scenedesmus obliquus CNW-N	0.1 M HCl, 0.1 M NaOH and 0.1 M $CaCl_2$	0.05 M $CaCl_2$	Cd(68.6 mg/g)	Cd(80%)	Cd(60 mg/g)	Na, 5	Chen et al. (2012)
Macrocystis pyrifera	HNO_3, EDTA, $Ca(NO_3)_2$	0.1 M HNO_3, 0.1 M EDTA	Zn(0.91 mmo/g), Cd(0.89 mmol/g)	Zn(99%), Cd(100%)	na	Na, 1	Cazon et al. (2012)
Microcystis	Distilled water, 0.5 M, 1 M, 2 M, 4 M, 6 M and 8 M of HCl	8 M HCl	Sb(III) 85%	Sb(III) 69%	Sb(III) 60%	Na, 5	Wu et al. (2012)

(White et al., 1998; Malik, 2004).

ESCALATION OF BIOSORPTION PROCESSES

Some of the reasons hindering successful implementation of biosorption processes at large scale has been discussed above. Innovative approaches gearing toward transformation of biosorption processes as realistic, reliable, sustainable, accessible and competitive industrial techniques for the treatment of heavy metal polluted waste wasters are still to be invented by researchers.

Efforts applied in the field of biosorption over four decades have not been satisfactorily translated into industrial applications (Tsezos, 2001; Volesky, 2007). Host of innovations patented since the early 80's have certainly contributed in their own right to the development of biosorption. From the understanding of the basic mechanisms of metal uptake by microbial sorbents, to the identification of suitable microbial sorbents, immobilization techniques, regeneration of biosorbents and biosorption process design, a lot has certainly being done to develop biosorption processes; however, there is still a long way to go as far as the implementation of an industrially competitive technique is concerned. Couple of biosorption processes have been tested at pilot scale and few have reached the stage of commercialization.

LARGE SCALE APPLICATION

More than ever, there is a crucial need for eco-friendly techniques capable to operate with natural resources using the minimum of power as possible. The enormous potential of biosorption technique for business opportunity (Volesky (2001) estimated the immediate and existing market for new biosorbents materials in North America to around US$30 million) is the major catalyst for the application and commer-cialization of biosorption-based products, however most of the attempts so far are premature, judging by the low percentage of successes achieved (Tsezos, 2001; Volesky, 2007; Wang and Chen, 2009). As from the early 1990's, the first proprietary biosorption processes or biosorbents were commercialized, and since then a number of attempts have been made to bring more to the market (Table 5). Several companies mainly from North America have developed immobilized biosorbents deriving from

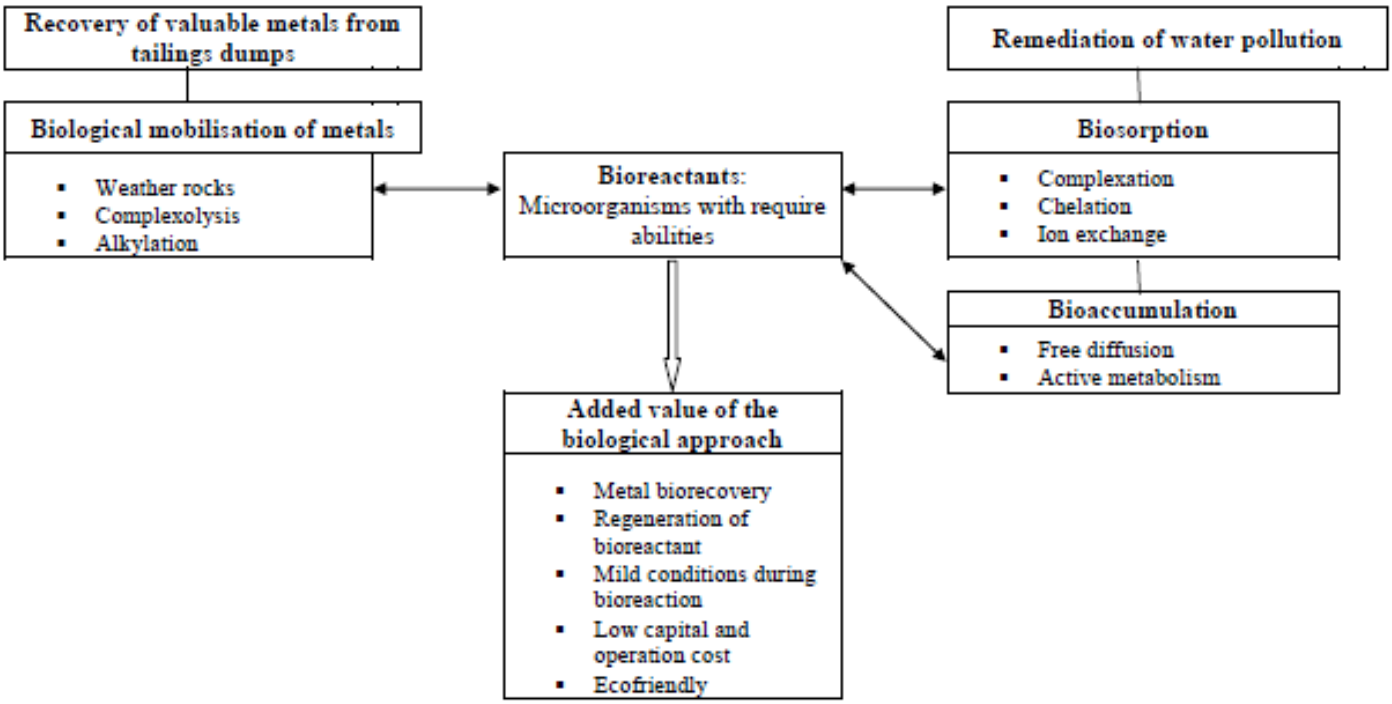

Figure 2. Illustration of hybridization of bioleaching and biosorption for prevention of metal dispersion

different natural raw materials; mainly from algae, yeast and bacteria biomasses. These biosorbents were proven to perform under harsh conditions such as extreme physic-chemical levels (pH, conductivity, temperature, etc.) and adsorbed mainly toxic heavy metals.

These biosorbents often have characteristics allowing great flexibility in engineering process design and applications. There is a number of metal-removal systems designed for industrial application of biosorption processes; these include fixed bed upflow and downflow reactors, fluidised beds, rotating biological contactors, trickle filters and air-lift reactors. Downflow systems are associated with some operational drawbacks, but the fact that they depend on gravitational forces to transfer the water body through the bed, provide a major advantage of lower power consumption over all the other systems requiring pumps or motors for water transfer (Atkinson et al., 1998; Volesky and Naja, 2005). On the basis of several parameters, Volesky and Naja (2005) found that fixed-bed continuous flow columns are the sorption system arrangement suitable for effective biosorption of heavy metals. According to Vijayaraghavan and Yun (2008), the design and type of process to be employed (batch/continuous) is entirely dictated by the choice of biomass and its method of immobilization.

The technological principles for industrial application of biosorption as elucidated above are similar for ion exchange; one could therefore expect that biosorption products will easily outperformed ion exchange products on the market, at least for the treatment of large volume of dilute effluents. However reports show that the ion exchange market is as well established as before and worse, the biosorption technology has been unable to sufficiently penetrate markets such as clean-up operation applications so far non-accessible to ion exchange process (Volesky, 2007; Wang and Chen, 2009). The limitations of industrial application of biosorption are well known and have been reported by several researchers (Tsezos, 2001; Wang and Chen, 2009):

i) Lack of reliable supply of waste microbial biomass suitable for specific application; fermentation industry was reluctant or unable to secure a steady supply of waste microbial biomass as the inexpensive raw material that would be used for production of the new biosorbents.

ii) The cost for producing the required biomass for the sole purpose of transforming this biomass into biosorbents was shown to be too expensive.

iii) The process has become more complex when considering distribution of immobilized biomass, regeneration, recycling and reuse of biosorbents.

iv) Challenges in technology development, researchers require the support of business partners for innovative process venture.

The knowledge and know-how acquired in the process of laboratories' investigations must be complemented with pilot studies and when needed, business expertise for successful commercialization of biosorption products.

Viability of industrial application

Enough care and investigations must be exercised to minimize the risk of failure and maximize the chances

Table 5. Examples of commercialised biosorption techniques.

Commercialized biosorbent	Family of biosorbent	State of cell free/immobilized	Company	Affinity	Application equipment	Reference
AlgaSORB™	*C. Vulgaris*	Immobilized on silica	Biorecovery Systems	Metallic cations and metallic oxoanions	Two columns operating in serie or in parallel	Chojnacka, 2010; Park et al., 2010; Wang and Chen, 2009
B.V. SORBEX	*S. natans, A. nodosum, Halimeda opuntia, Palmyra pamata, Chondrus crispus and C. Vulgaris*	Powder or granules	BV SORBEX Inc	Specific to toxic heavy metals	Fixed bed system, fluid bed system and completely mixed tanks	Park et al., 2010; Wang and Chen, 2009
AMT-BIOCLAIM™	Bacillus treated with caustic soda	Immobilized in extruded beads-polyethyleneimine and glutaraldehyde		Suitable for accumulation of gold, cadmium and zinc from cyanide solutions	Fixed bed canisters or fluid-bed reactor systems	Chojnacka, 2010; Park et al., 2010; Wang and Chen, 2009
BIO-FIX ®	Sphagnum, peat moss, algae, yeast, bacteria and aquatic flora	Immobilized in polysulfone	U.S. Bureau of Mines (Golden Colorado)	Selective for toxic heavy metals		Chojnacka, 2010; Park et al., 2010; Wang and Chen, 2009
MetaGeneR				Remove heavy metals from electroplating and mining waste streams		Chojnacka, 2010; Park et al., 2010; Wang and Chen, 2009
RAHCO Bio-Beads	Variety of sources including peat moss	Immobilized within an organic polymer		Remove heavy metals from electroplating and mining waste streams		Chojnacka, 2010; Park et al., 2010; Wang and Chen, 2009

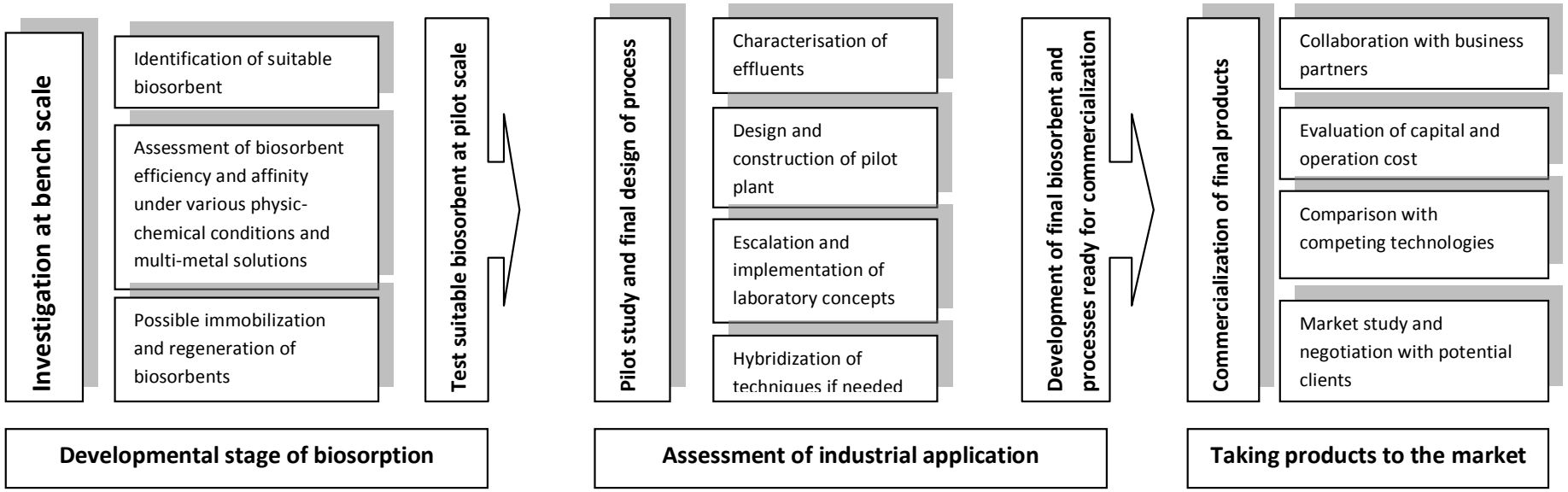

Figure 3. Schematic representation of the pathway of biosorption development.

for successful commercialization of biosorption products. In the process of escalating biosorption process, technical aspects related to the possible application of laboratories' concepts to the treatment of real industrial effluents have to be carefully investigated by constructing pilot plants (Figure 3). Points to be considered during such investigation were extensively discussed by other researchers (Atkinson, 1998; Volesky, 2007; Vijayaraghavan and Yun, 2008; Wang and Chen, 2009) and have to do with the informed selection of biosorbent based on the characteristic of the effluents including the nature of pollutants, the cost and the availability of biomass.

A very important point that requires collaboration with a business partner is the knowledge of the overall commercial potential of the biosorption technology. Strategic approach in positioning biosorption products on the market must be taken, prioritizing the focus on the market share of biosorption technology and the advantages of this new technology over the existing ones. Practically, information on the possibility for biosorption technology to penetrate the market can be acquired by conducting studies based on:

i) Comparison of the cost of the new biosorbent-based technology with the cost of conventional technology for the treatment of a given solution.
ii) Determination of the approximate figure of the market size requiring ecofriendly treatment of metal polluted effluents.

It is important to mention here that, the main struggle with biosorption is undoubtedly at the technological level. There is still a need to develop a finish product (that may include hybrid biological technologies) capable to effectively remedy heavy metal pollution of effluents at competitive cost. To a large extend we have not reached that stage and most of the attempts of commercialization are premature.

CONCLUSION

It is very important for all those who envisage escalating the biosorption processes for comercialization, not to consider applying biosorption as a "unique solution for decontamination of all pollutants in the effluent". One must bear in mind the potential of the biosorbents as well as their limitations. Environmental effluents have a complex physic-chemical characteristic often containing a multitude of pollutants likely to inhibit the action of the biosorbents. The capacity of biosorbents to remove metals is unequivocal, capable and excellent biosorbents have been identified

and developed for metal removal and only require to be used under suitable conditions. Characterization of effluents and involvement of hybrid technologies if needed for the pretreatment of effluents must be considered upfront. There is a growing demand for ecofriendly and cheap technologies, hence a huge portion of the market which cannot be covered by conventional technologies; the bottom line is cheap, reliable, effective and an industrial processes (possibly a hybrid biological system involving biosorption) for treatment of toxic metals polluted effluents developed on the basis of effluents characteristics and tested accordingly in pilot plants prior to consideration for commercialization.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Micropropagation of an endangered medicinal herb *Chlorophytum borivilianum* Sant. et Fernand. in bioreactor

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Chlorophytum borivilianum **Sant. et Fernand. is an endangered herb, the tuberous roots of which are source of medicinally important steroidal saponins. In the present study, propagation of *C. borivilianum* using a bench top stirred bioreactor with liquid medium via multiple shoot culture has been reported. One week old shoots along with shoot base part (1.5 cm) obtained from shoots regenerated *in vitro* in liquid medium shake flasks containing 22.2 µM 6-benzylaminopurine, were used as explants. An inoculum density of 120 explants/2.5 L liquid Murashige and Skoog medium supplemented with 22.2 µM 6-benzylaminopurine was found optimal for shoot growth. After three weeks of culture, 4.4-fold increase in biomass (fresh weight) was obtained. Shoots regenerated in bioreactor were rooted *ex vitro* on three-fourth strength liquid MS medium supplemented with 9.8 µM indole-3-butyric acid. Plantlets with 100% rooting of microshoots were hardened and established in the glasshouse with 85% survival rate. Due to rapid and efficient propagation in bioreactor with high survival rate, this protocol may be employed for conservation and large-scale multiplication of *C. borivilianum*.**

Key words: Bioreactor, *Chlorophytum borivilianum*, hyperhydricity, saponins, shoot culture.

INTRODUCTION

Chlorophytum borivilianum commonly known as safed musli (family Liliaceae) is a monocotyledonous plant. Tuberous roots of safed musli contain steroidal saponins (neohecogenin, neotigogenin, stigmasterol, tokorogenin) that are used as tonic and aphrodisiac (Kaushik, 2005; Tandon et al., 1992; Deore and Khadabadi, 2010). Saponins also possess anti-tumour activity (Kumar et al., 2010). They are used in the industrial production of sex hormones, corticosteroids and steroid derivatives. The roots of the plant are widely used in the Indian system of medicine for the treatment of weakness, impotency and sterility; to enhance male potency; as cardiac and brain

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tonic; as a curative agent in various diseases like piles, diabetes and as diuretic and hemostatic. *C. borivilianum* has been used along with other plants such as *Asparagus adscendens*, *A. racemosum*, *Curculigo orchioides* and *Withania somnifera* in many herbal and Unani formulations (Thakur et al., 2009; Ramawat et al., 1988). Due to its therapeutic activity and diversified uses, there is demand for safed musli especially *C. borivilianum* in India. Many countries in Gulf, Europe and USA have been major importers of the dry roots of safed musli for preparation of various herbal products in pharmaceutical, phytopharmaceutical and nutraceutical industries. While, the global demand for dry safed musli has been estimated in the order of 35,000 tonnes per annum, the supply stands at 5,000 tonnes per year (www.farmwealthbiotech.com, 2010). Safed musli is a rhizomatous herb propagated vegetatively in nature by shoot buds from perennating root tubers in the soil and sexually by seeds. Seeds have poor germination percentage (11 to 24%), low viability and long dormancy period leading to scarcity of tuberous roots of the plant in nature (Bordia et al., 1995). Conventional techniques for vegetative propagation of planting material are slow and cannot keep pace with the present demand (Bordia et al., 1995). About 95% of the indigenous requirement comes through wild habitats. Due to large-scale and indiscriminate collection of wild material and insufficient attempts to allow its replenishment, *C. borivilianum* is rapidly disappearing. Besides, the scarcity of elite and characterized planting material and non-availability of improved agro technological practices have also limited its commercial cultivation. There is need for conservation and mass multiplication of this plant. The plant has been enlisted as an endangered plant species in Red Data Book of India (Nayar and Sastry, 1988) and National Medicinal Plant Board (NMPB), India has categorized it as one of the prioritized plant species to be promoted for conservation and large-scale cultivation and export of this herb is being actively encouraged by Government of India through NMPB. Therefore to fill the gap between demand and supply of its tuberous roots to meet Industry's requirement and to provide cost-effectiveness, genetically uniform planting material for conventional propagation at a rapid rate in a short span of time, alternate propagation strategies such as micropropagation are urgently needed which will lead towards conservation and mass production of the plant.

Micropropagation serves as a means of clonal propagation of economically important species and as a tool for germplasm conservation. Micropropagation technology is advantageous due to production of high-quality disease-free, true-to-type plants independent of seasonal and other environmental conditions in a comparatively smaller space (Gurel, 2009), but commercial micropropagation is largely limited by the higher cost of plant production (Kozai et al., 1997). Liquid culture was reported to be ideal in micropropagation for automation and reduced manual labour (Roels et al., 2006) and for reducing plantlet production cost (Scheidt et al., 2009). Many plants have been mass-propagated in the liquid medium using bioreactors (Mehrotra et al., 2007). Automation of micropropagation in bioreactors has been advanced as a possible way of reducing cost of micropropagation (Son et al., 1999; Ibaraki and Kurata, 2001; Paek et al., 2001). Various types of simple bioreactors with mechanical or gas-sparged mixing were used in plant and cell cultures to provide stirring, circulation and aeration (Paek et al., 2005; Yesil-Celiktas et al., 2010). Mechanically stirred bioreactors depend on impellers, including a helical ribbon impeller, magnetic stirrers or vibrating perforated plates (Archambault et al., 1994). Aeration, mixing and circulation in bubble-column or airlift bioreactors are provided by air entering the vessel from the side or from the basal opening through a sparger. As the air bubbles rise, they lift the plant biomass and provide the required oxygen (Preil, 1991). Oxygen (O_2) requirements may vary from one species to another and concentration of O_2 in liquid cultures in bioreactors can be regulated by agitation or stirring and through aeration, gas flow and air bubble size. The other key parameter is mixing, which is necessary to distribute cultured cells or tissues and nutrients equally throughout the liquid phase (Honda et al., 2001). Mixing is normally carried out by sparging, mechanical agitation or a combination of these two, but the magnitude of hydrodynamic forces associated with mixing should be small enough not to cause cell or tissue damage, but sufficient to stimulate desired cell/tissue growth. In the present study, *in vitro* propagation of *C. borivilianum* in a bench top stirred bioreactor of 5 L working capacity, employing liquid medium has been reported for scaling-up cost-effective mass propagation of the plant.

MATERIALS AND METHODS

Bioreactor configuration

In the present study, experiments for large-scale culture of *C. borivilianum* were done by using a bench top stirred bioreactor (Bio Flow-110, M/s New Brunswick Scientific, USA) having working volume of 5 L. The impeller in this air sparged top driven system provided mechanical agitation (75 rpm). The bioreactor consisted of a thick glass jacketed vessel assembly having inner vessel with removable baffle assembly consisting of different probes including dissolved oxygen (DO), pH and temperature probes to control and optimize various culture conditions. The inner vessel assembly was surrounded by an outer thick glass jacket. The external jacket was used as a water temperature controller. The whole glass culture vessel rested on a double walled stainless steel base through which cold water (4°C) was circulated, which maintained the temperature of the culture medium. An exhaust gas condenser was installed to the unit for minimizing the medium evaporation during culture period. All parts of the bioreactor assembly were thoroughly washed and then surface sterilized with ethyl alcohol prior to assembling the unit and lubricated with silicone grease to make the unit air tight. About 2.5 L liquid (without agar), Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented

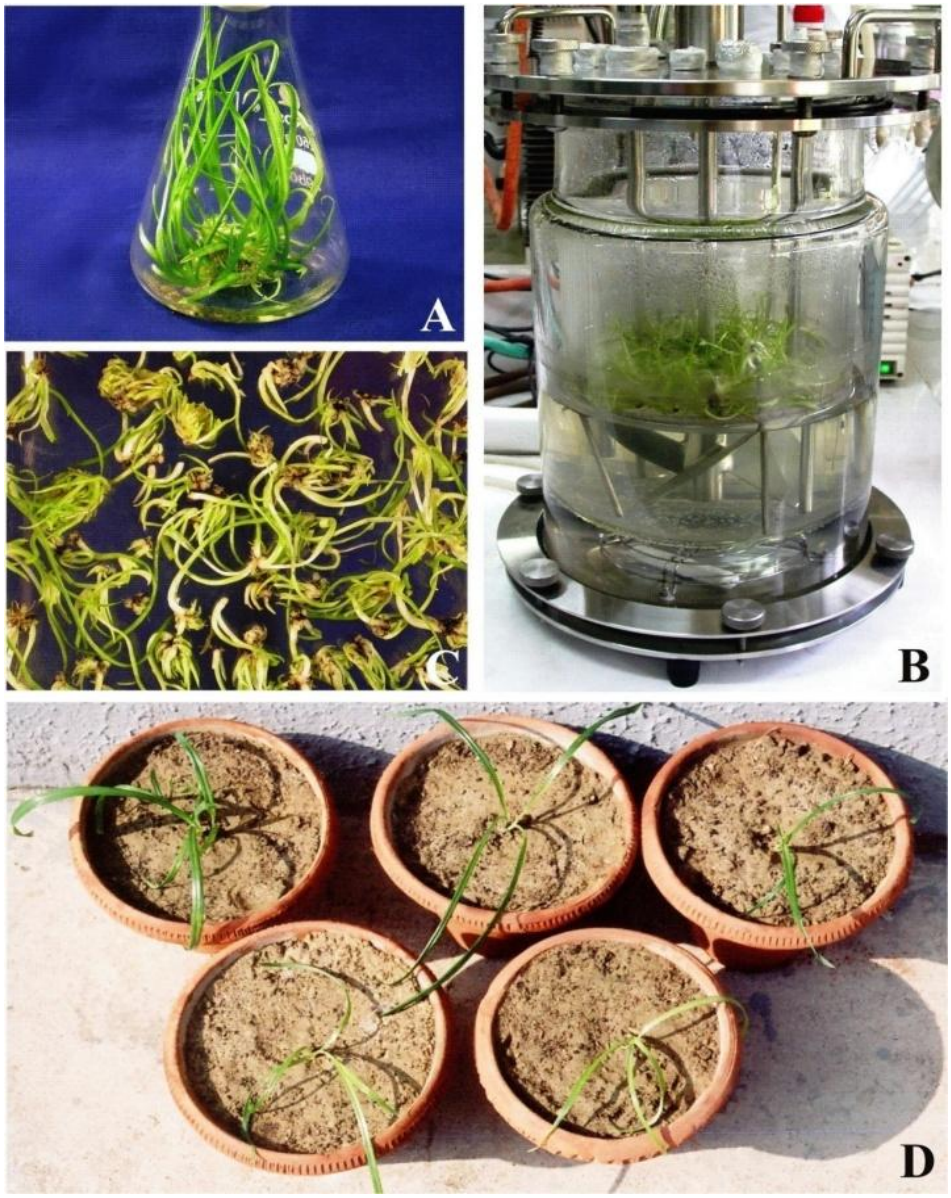

Figure 1. Propagation of *C. borivilianum* in bioreactor: *in vitro* regenerated shoots in liquid medium shake flasks from which explants were obtained (A), shoots cultured in bioreactor (B), shoot biomass harvested from bioreactor after 3 weeks of culture (C) and plants raised from bioreactor culture in earthen pots after 10 weeks of hardening (D).

with 22.2 µM 6-benzylaminopurine (BAP) and 3% (w/v) sucrose were poured in the culture vessel. The pH of the medium was adjusted to 5.8 ± 0.1 using 0.1 N HCl or 0.1 N NaOH prior to autoclaving. Earlier, experiments amongst a wide range of BAP (2.2 to 40.0 µM) tested for their efficacy on *in vitro* shoot multiplication, BAP at 22.2 µM level exhibited optimal response. During the experiments, an autoclavable nylon mesh (pore size 200 µM) tightened just beneath the surface of culture medium on the lower stainless steel semi-circular ring of the baffle assembly prevented the explants from sinking to the bottom or getting submerged in the medium (Figure 1 B). The explants thus remain floating near the medium surface with the support of mesh during the culture period and therefore, they will not become completely immersed in the medium. The incoming air was passed within the medium at 0.5 L/min rate through air sparger after sterilization by a hydrophobic sterile membrane filter (Whatman, USA, 0.22 µ). The complete bioreactor unit was properly assembled and autoclaved at 121°C and 15 lb pressure for 25 min in a vertical cylindrical sterilizer (M/s Yarco, India).

Inoculation of bioreactor vessel

Explants, consisted of one week old shoots along with shoot base part (1.5 cm in length) obtained from shoots regenerated *in vitro* in liquid medium shake flasks (Figure 1A), were aseptically transferred from Erlenmeyer flasks containing MS liquid medium supplemented with 22.2 µM BAP to the sterilized bioreactor culture

Table 1. Shoot regeneration and growth of *Chlorophytum borivilianum* shoot base explants cultured in bioreactor for 3 weeks.

Volume of medium (L)	Inoculum density		Shoot growth response (%)	Final f.wt. of harvested biomass	Percentage increase (biomass)
	Number of explants inoculated	Total f.wt. (g) of the inoculums			
2.50	120	11.90	75	52.36	4.4-fold

f.wt. = fresh weight.

vessel containing same liquid culture medium. Mother plants were obtained from natural habitat (Rajsamand District in the State of Rajasthan). Amongst these mother plants, a high yielding line of *C. borivilianum* selected on the basis of root yield, was maintained in Botanical Garden of the Institute. *In vitro* shoot cultures were established from young shoot apices obtained from the tuberous roots of this selected high yielding line. Approximately, 120 explants (inoculum density) having fresh weight of about 11.90 g were aseptically transferred from Erlenmeyer shake flasks to the sterilized bioreactor through its inoculation port. After inoculation, bioreactor culture vessel was installed on the main control module. Water lines were connected to the water jacket inlet and to the exhaust condenser. All air or gas lines were also reconnected. The pH and DO cables were connected to their respective probes and to the pH and DO control module. The temperature probe was reinstalled into the thermowell. Air sparger was connected to air compressor through silicone tubing fitted with 0.22 µ sterile microfilter (Whatman membrane filter, USA). Culture conditions included 16:8 h light: dark alternating regime of 45 µmol m^{-2} s^{-1} intensity provided by cool white fluorescent tubes, 25 ± 1°C incubation temperature and 50 to 60% relative humidity. Final shoot biomass (fresh weight) and increase in biomass (fresh weight) over initial biomass (fresh weight) were recorded after 3 weeks. The experiment was repeated thrice. Observations are mean of three experiments.

Rooting and acclimatization of *in vitro* regenerated plants

Shoots (3 to 4 cm) regenerated *in vitro* in bioreactor after three weeks of culture of shoot base explants on MS medium supplemented with 22.2 µM BAP, were washed with water and placed at the top of culture tubes filled with three-fourth strength MS nutrient solutions supplemented with 9.8 µM indole-3-butyric acid (IBA) or three-fourth strength MS medium without any growth regulators (control) for *ex vitro* rooting. The shoots regenerated in bioreactor were also kept for *in vitro* rooting on the same culture media as in case of *ex vitro* rooting. The 9.8 µM IBA level was found optimal for rooting of *C. borivilianum* shoots amongst various levels (0.2 to 24.6 µM) of IBA added to three-fourth strength MS liquid medium in the previous experiments. The culture tubes containing shoots regenerated *in vitro* in bioreactor were kept in the culture room for 3 weeks under similar culture room conditions as described above for rooting and then rooted plants were transferred to earthen pots consisting sand: soil: farmyard manure in 1:1:1 (v/v) ratio. Potted plants were covered with transparent polythene bags with small holes for air ventilation to ensure high humidity in initial stages. The polythene bags were removed after 2 to 3 weeks and the surviving plants were maintained in the glass house under 16 h photoperiod at 28 ± 2°C. Number of roots, rooting percentage and root length were recorded after 3 weeks of culture on IBA containing MS nutrient medium as well as on MS medium without growth regulators. The survival percentage was recorded after 12 weeks of transfer of plants in pots.

RESULTS AND DISCUSSION

Up-scaling of shoot cultures of *C. borivilianum* in bioreactor

After 3 weeks of incubation period, the growing shoots were harvested and growth response was recorded (Figure 1C). About 75% response in terms of shoot regeneration and growth was observed and biomass yield of 52.36 g (fresh weight) was recorded after 3 weeks while initial biomass (fresh weight) of all inoculated explants was 11.9 g. Thus, 4.4-fold increase in biomass (fresh weight) over the initial inoculum biomass (fresh weight) was observed in the present investigation after 3 weeks of culture period (Table 1). Large-scale plant tissue culture using bioreactors is promising for industrial plant propagation. It offers various advantages including controlled supply of biochemicals independent of plant availability (cultivation season and pests), consistent quality of the product and it also overcomes the drawbacks of plant cell culture systems. *In vitro* tissue culture using bioreactors enables the production of genetically identical individuals from an elite plant and allows plant multiplication, free of pathogens in less time as compared to conventional methods. The use of liquid culture decrease the plantlet production cost (Scheidt et al., 2009) and they are ideal for automation. Many plants especially medicinal plants have been mass-propagated in the liquid medium using bioreactors (Paek et al., 2005; Mehrotra et al., 2007).

In the present study, the explants size of 1.5 cm resulted in the high shoot growth in bioreactor. While in the case of apple rootstock, the explants size of 0.5 cm or 1 cm resulted in higher shoot production rate as compared to explants size of 1.5 cm (Zhu et al., 2005). The inoculum density in the present investigation was 120 explants in 5 L bioreactor which resulted in high shoot growth in *C. borivilianum*. While optimum inoculum density in a 3 L bioreactor was 200 explants for rhizome multiplication and shoot differentiation in *Cymbidum niveo-maginatum* (Jin et al., 2007). In the present study, high shoot growth was obtained on full strength MS medium supplemented with 22.2 µM BAP. On the other hand, differentiation of *Lilium* bulblets on bulb scales, cultured in bioreactors, was better on half-strength than

on full strength MS medium (Takayama, 1991).

In the present study, in a bench top stirred bioreactor employing liquid medium with helical ribbon impeller and gas sparged mixing, biomass increase of 4.4-fold was obtained. The presumed reasons for faster growth in the liquid medium were thought to be better availability of nutrients and rapid uptake of nutrients. A biomass increase of six-fold was obtained without need for periodic transfer of explants to fresh medium in *Vaccinium corymbosum* (Ross and Castillo, 2009). This represents an effective reduction of cost, where intensive manual handling is the main component. Also, during entire culture period of 3 weeks of *C. borivilianum* in bioreactor, there was no periodic transfer of explants/growing shoots to fresh medium thus removing the need for additional manual labour resulting in cost-reduction. An additional reduction in cost derived from the use of liquid medium thus avoiding the use of agar which otherwise adds up to 65% of the cost of the culture medium (Mitra et al., 1998). However, the use of liquid media in bioreactors can lead to the problem of asphyxia and hyperhydricity in explants as a result of immersion. Hyperhydricity induces morphogenic abnormalities in the developing plantlets (Haq and Dahot, 2007) and subsequently affect their survival. Attempts to control hyperhydric deformities have been largely focussed on better aeration and intermittent or partial plant submergence in the medium using temporary immersion bioreactors (Sajid and Pervaiz, 2008; Farahani and Majd, 2012). In the present study, the explants/growing shoots were supported by nylon mesh in the way that their lower part was continuously immersed in the culture medium, on the other hand, upper portion was exposed to air thus avoiding hyperhydricity of the growing shoots and also preventing sinking of the explants/growing shoots to the bottom of the culture vessel. Similar with our observations, shoot growth and subsequently growth of plantlets in *Anoectochilus formosanus* was more efficient in continuous immersion (with net support) bioreactor as compared to continuous immersion (without net support) and temporary immersion bioreactor culture in liquid media using ebb and flood (Wu et al., 2007). Shoot growth and proliferation were most efficient in balloon type bubble bioreactor (BTBB) having continuous immersion culture with a net to avoid the complete immersion of plant material in garlic as compared to BTBB (immersion culture without net and ebb and flood culture), (Kim, 2002).

In the present study, principles of both mechanically stirred and gas sparged bioreactors were employed. Aeration and circulation were provided by filtered sterile air at the rate of 0.5 L/min throughout the culture duration by two spargers, one above the autoclavable nylon mesh and another just below the mesh and mechanical agitation was provided by a helical ribbon impeller at the rate of 75 rpm for mixing of nutrients and plant growth regulators throughout liquid phase. The aeration at the rate of 0.5 L/min and agitation at 75 rpm were found optimal for the shoot growth in the present investigation. Lack of oxygen in the liquid media containing small explants and asphyxia and hyperhydricity of explants as a results of immersion, are the major limiting factors to their growth (Farahani and Majd, 2012). Supply of compressed air inside the bioreactor chamber for decreasing humidity significantly reduced the hyperhydricity during the bioreactor culture of apple root stock 'M9 EMLA' plantlets (Chakrabarty et al., 2003). Also, supply of filtered sterile air through sparger at 0.5 L/min rate was found to be helpful in reducing hyperhydricity in *C. borivilianum*.

Rooting

In the present investigation, shoots obtained from the bioreactor after 3 weeks of culture, 3 to 4 cm long were rooted *ex vitro*. Shoots kept in the MS medium devoid of growth regulators (control) developed roots at a rate of 60% while shoots in 9.8 µM IBA-supplemented medium showed 100% rooting with 5 to 6 roots per plantlet. The length of roots was 3 to 4 cm. On the other hand, in *in vitro* rooting of microshoots of *C. borivilianum*, 40 and 80% rooting percentage were observed respectively on control medium (MS medium without growth regulators) and IBA supplemented medium which is lower than rooting percentage of *ex vitro* rooted microshoots. Sugarcane shoots regenerated in bioreactor were also rooted *ex vitro* on MS medium supplemented with 2.5 µM IBA and rooting response was noticed in three weeks with a success rate of 83% (Sajid and Pervaiz, 2008). On the other hand, in mass propagation of *V. corymbosum* in bioreactor, 80% rooting was observed *ex vitro* in the control treatment without IBA. While, exogenous addition of IBA resulted in inhibition of root differentiation (Ross and Castillo, 2009). In the present study on *C. borivilianum* micropropagation in bioreactor, roots regenerated *in vitro* were fragile while *ex vitro* derived roots were thick and have a good root system. The survival percentage of plantlets having *ex vitro* rooted shoots was 85% as compared to 70% in *in vitro* rooted shoots. These results show that bioreactor-derived microshoots of *C. borivilianum* are suitable for *ex vitro* rooting thus avoiding the need for *in vitro* rooting which is more time and labour-consuming. Consistent with our investigation, in the studies of Borkowska (2001) on micropropagated strawberry shoots, the *in vitro* formed roots were fragile and easily damaged. Plantlets that were rooted *ex vitro* had a larger root system and more runners than those formed by *in vitro*-rooted strawberry plantlets. The 60% rate of *ex vitro* rooting on growth regulator free medium may add towards the cost-effectiveness of rooting of bioreactor derived *C. borivilianum* microshoots.

Hardening and transplantation

Shoots regenerated in bioreactor with well developed roots after 3 weeks of growth on three-fourth strength MS

medium supplemented with 9.8 µM IBA, were transferred to glasshouse. *Ex vitro* rooted plantlets acclimatized to the glasshouse with survival rate of 85% (Figure 1D), while the survival percentage of plantlets having *in vitro* rooted shoots was 70%. Performance of plants established in glass house was uniform. The plants showed green colour and without mutations. In the studies of McGranahan et al. (2006), due to *ex vitro* rooting of microshoots of walnut rootstock genotypes, survival of rooted microshoots was improved to 80% for *ex vitro* rooted plantlets as compared to 50% for *in vitro* rotted ones of the same genotypes. *Ex vitro* rooted plantlets grew faster in the greenhouse. In low bush blueberry, micro cuttings regenerated in bioreactor performed well in the greenhouse and rooted plants have survival rates of 90 to 99% (Debnath, 2009). While, in the present investigation; *C. borivilianum* plants regenerated in bioreactor and rooted *ex vitro* exhibited 85% survival rate.

Conclusion

In conclusion, this report presents a protocol for *C. borivilianum* micropropagation for the first time in a bioreactor system. About 75% response in terms of shoot regeneration and growth and 4.4-fold increase in biomass (fresh weight) was observed in the present investigation after 3 weeks of culture. The *ex vitro* rooting of bioreactor-derived microshoots of *C. borivilianum* is important in economic sense as it is more cost-effective, less time and labour-consuming as compared to *in vitro* rooting. The rooting at the rate of 60% on growth regulator free medium may be important in cost-effectiveness of micropropagation of *C. borivilianum* in bioreactor. Thus, due to rapid and efficient shoot proliferation, biomass increase, efficient rooting and successful transfer to glasshouse with 85% survival rate, this protocol can be used for large-scale propagation and conservation of this important medicinal plant.

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Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Utilization of seafood processing wastes for cultivation of the edible mushroom *Pleurotus flabellatus*

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A study was conducted to examine the utilization of seafood processing wastes for artificial cultivation of edible mushroom *Pleurotus flabellatus* in laboratory condition. Utilizing bioconversion technology such as the slow release of nutrients for agricultural based activities like producing mushroom will profitably reduce seafood waste and also enhancing environmental quality. The selected agro-industrial wastes such as coir pith, woodchips and sugarcane bagasse were mixed with cooked fish waste (CFW) in the ratio of 1:1 (500 g : 500 g). The substrates which were not mixed with CFW were treated as control. All the above materials were allowed to decompose partially for about 15 days. The partially composted materials were placed in heat resistant transparent sterilized polyethylene bags. Each sterile bag was then aseptically inoculated with *P. flabellatus*. The bags were then incubated under ambient temperature and controlled humidity. The maximum biological yield per bed was obtained with sugarcane bagasse control bed 58.05±0.88 g/bed. The lowest yield was observed in the substrate woodchips: CFW (1:1) 24.43±0.30 g / bed. Based on the mass obtained for *P. flabellatus*, the best substrates were in the ordered of woodchips>coir pith>sugarcane. This could be used to cultivate an edible mushroom while at the same time promoting environmental sustainability and increase soil fertility.

Key words: Mushroom, cooked fishery waste, solid substrates, biological yield.

INTRODUCTION

Seafood processing activities have raised serious waste production and disposal concerns all over the globe. Commercially, fishing and aquaculture usually generate large amounts of waste that must be disposed (Burrows et al., 2007). In Korea, many restaurants specialize in sliced raw fish, and large amounts (approximately 2,100 t/day) of fish waste are generated every day (Kim et al., 2010). Fish consumption continues to increase steadily worldwide and seafood is gaining in popularity because of its health benefits. At the same time, large amounts of fish waste are being generated, mostly from the industrial processing of fish. These large quantities of fish waste

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have not been utilized efficiently, and the disposal of fish waste can have large negative impacts on local environments. Unutilized fish waste is often disposed of by landfill or incineration, or by dumping into the sea. Therefore, there is an urgent need to find ecologically acceptable means for reutilizing fish waste (Joong, 2011).

The fermented broth of fisheries waste could be a valuable resource for agriculture. Recently, some studies have examined the reutilization of biodegraded fisheries-waste products as liquid fertilizer (Kim and Lee, 2009; Kim et al., 2010; Dao and Kim, 2011). Fish sludge contains macro and micro nutrients, especially high levels of nitrogen and phosphorus. Sewage sludge mixed with different organic waste materials is now usual in composting experiments (Roca-Perez et al., 2009).

The "Mushroom" word is used in all part of world to describe the fruiting bodies of saprophytic, mycorrhizal and parasites fungi, belonging to the order of Basidiomycetes or Ascomycetes. They can be found in soils rich in organic matter and humus, moist wood, animal waste, etc. The cultivation of edible mushrooms is actually an alternative biotech which is fast, environmentally friendly and feasible to recycle organic byproducts from agribusiness into high nutritional and medicinal quality food both with respect to the amount of protein or minerals and selected substances with medicinal and pharmacological properties, for example the presence of β-glucans like lentinan, and thus it can contribute significantly in feeding human. (Diego Cunha Zied et al*.,* 2011).

Cultivation of edible mushroom using various wastes is an alternative for solid waste management. Mushrooms have very high nutritional value. It is rich in protein, vitamins and minerals. More than 2000 species of fungi are reported edible throughout the world and 200 belonging to 70 genera are reported from India. Of these, about 80 distinct varieties which are edible are sold in various countries. Presently, only three mushrooms namely *Agaricus bisporus* (white button mushroom), *Pleurotus* spp. (oyster mushroom) and *Volvariella volvacea* (Paddy straw mushroom) are under commercial cultivation in India. *A. bisporus* with its temperature preference could not be cultivated in tropical and sub tropical situations leaving the choice to only *Pleurotus* spp. (Renganatha et al*.,* 2008).

Pleurotus species are rich source of proteins and an abundance of essential amino acids, minerals (Ca, P, K, Fe, Na) and also contain vitamins C, B-complex – thiamine, riboflavin, niacin and folic acid (ÇaglarIrmak, 2007; Regula and Siwulski, 2007). Oyster mushroom *Pleurotus ostreatus*, due to its documented probiotic properties and relatively high nutritive value, are recommended in numerous countries as an addition to the daily diet (Bernas et al., 2006).

Successful utilization of agro-wastes for both mycelial and sporophore formation of macrofungi, supplies the nutrients needed by these fungi to convert them to protein-rich palatable food. It also helps in reducing the environmental and health hazards posed by indiscriminate dumping of the wastes (Pandey, 2006). Mushroom hyphae secrete large amounts of extracellular enzymes which bring about the degradation of macromolecules such as cellulose, hemicellulose, lignin and protein in the substrates (Narsi et al., 2006; Kuforiji and Fasidi, 2008).

Oyster mushroom can be grown on various substrates (Hassan et al., 2011). Cultivation of edible mushrooms is a biotechnological process for lignocellulosic organic waste recycling. Roughly 300 mushrooms species are edible, but only 30 have been domesticated and ten grown commercially (Barny, 2009). *Pleurotus* sp. is the second most cultivated edible mushroom worldwide after *Agaricus bisporus.* To date approximately 70 species of *Pleurotus* have been recorded and new species are discovered more or less frequently (Ruhl et al., 2008). These mushrooms have economical and ecological values and medicinal properties. They are able to colonize and degrade a large variety of lignocellulosic substrates and other wastes which are produced in agricultural, forest and food processing industries (Sanchez, 2010).

Pleurotus pulmonarius and *Pleurotus ostreatus*, exhibited strong anti-inflammatory and immunemodulatory properties due to their chemical composition (Lavi et al., 2010). In the present study using *Pleurotus flabellatus* an attempt has made to convert fishery waste in to the valuable organic manures.

MATERIALS AND METHODS

Tissue culture technique

A large healthy and fresh mushroom *P. flabellatus* were collected from the Agricultural College at Killikulam, Tirunelveli District, Tamil Nadu and India. It was cleaned with 75% alcohol. The mushroom was splitted in half by hand longitudinally and some inside tissue taken from the upper part of the stripe. It was placed centrally on the surface of the sterilized potato dextrose agar with a sterilized needle and kept at room temperatures (28 ± 2°C) for eight days. Within two or three days some white, delicate mycelia was produced from the small piece of the tissue. About eight days later, the mycelium had grown rapidly and covered the surface of the agar medium. The growth of mycelium around the tissue inserted without contamination was considered as positive growth. The pure culture were collected and stored in the slants at 4°C for a period of a month. Then, it was ready to transfer to spawn substrate to make spawn. This spawn was used as inoculums for cultivation of mushroom (Stamets, 2000; Dhouib et al., 2005).

Spawn production

Spawn preparation was done using the *Shorgum vulgare* grains. The *S. vulgare* grains (1 kg) were placed in a trough of water to remove the chaff grains. Then, it was half cooked (~ 30 min). The excess water were drained and spread over a clean Hessian cloth. For every kilogram of grain, 20 g calcium carbonate was mixed so as to maintain the pH for the growth of the fungus. Moreover, the

calcium carbonate coating prevented the grains from sticking. These grains were filled in clean polythene bags (300 g/bag). Then, the bags were tightly plugged with nonabsorbent cotton and wrapped with paper, tied with a thread and placed in an autoclave for sterilization (20 lbs pressure for 2 h). After cooling, the bags were ready for inoculation. With the help of the gel puncher, a 10 mm diameter disc was made on the Petri plates having fully grown pure mycelium and transferred to the spawn bags. The bags were incubated at the room temperature. In about 15 days, the white colored mycelial growth was spread over the bag (Mane et al., 2007; Vetayasuporn et al., 2007; Royse et al., 2004; Shah et al., 2004; Baysal et al., 2003; Obodai et al., 2003a; Stamets, 2000). From this mother spawn, 30 first generation spawn were prepared. Each first generation bag having 10 g of mother spawn and from this second generation spawns were prepared.

Compost of fish waste

Fishery wastes were collected from the food processing unit at Thoothukudi District, which contains head, tail, shells, intestine, fins, dead fishes and so on. The wastes obtained were brought to the laboratory and the uncompostable materials such as shells and large bones were removed. The remaining wastes were cut into small uniform size pieces and allowed to cook for 15 min. Disease-free agro-industrial wastes (sugarcane baggase, coir pith and woodchips) were collected from Thoothukudi district which were cut in to small pieces (2 to 3 cm) and sun-dried in order to achieve proper drying. The selected agro-industrial wastes were mixed with cooked fish waste (CFW) in specific ratio 1:1 (500:500 g); while those which were not mixed with CFW are regarded as control. All the above materials (1:1 and control) were heaped in a separate plastic container and allowed to decompose for about 15 days. During the decomposition, water was sprayed over the materials with turning and restacking for every day to produce homogenize composed. Finally, the partially fish compost (15^{th} day compost) materials were used for mushroom cultivation.

Preparation of *P. flabellatus* bed

The partially fish compost (15^{th} day compost) materials were filled in polythene bags and sterilized at 121°C for 2 h. After sterilization, the bags were cooled to room temperature. Then polythene bag of 30×15 cm size was taken and the bottom of the bag was tied with a thread to provide a flat circular bottom to the mushroom beds. The partially fish compost (15^{th} day compost) materials were transferred to these polythene bags carefully along with previously prepared experimental spawns. The spawning was done in four to five layers. One bottle of spawn was used for two experimental bags. Then, the mouth of the bag was tied with the help of a twine and air holes were made on the sides of the bags for the free flow of air in to the mushroom bed. These polythene bags were kept in the dark room at 25 to 30°C. Relative humidity in the room was maintained as 86±4% and temperature as 26.5±0.05°C, respectively by pouring 25 L of water per day on the floor and on the walls. Whenever is necessary, the moisture content of the bags were maintained by the mist sprayers. The matured *Pleurotus* species fruiting bodies were identified by the formation of curl margin of the cap. It was then harvested from the root from the base by using a sharp sterilized knife. Mushroom matured generally 48 h after the appearance of primordia. Data on period of after completion of mycelium running, days of first harvest, number of fruiting bodies, length, diameter, biological yield, biological efficiency, moisture content of mushroom and dry yield were recorded. The biological efficiency (BE) percentage [fresh weight of harvested mushrooms/dry matter content of the substrate] x 100 (Royse et al., 2004; Stamets, 2000).

Data collection

Data on the following parameters were collected following the standard procedures (Ashrafuzzaman et al., 2009).

Time required for completion of mycelium running

Day required from opening to primordial initiation and days required from opening to harvesting on different substrates were recorded.

Number of fruiting body and dimension of pileus

Number of well-developed fruiting body was recorded. Dry and pinheaded fruiting body was discarded but twisted fruiting body was included during counting. Thickness of the pileus of four randomly selected fruiting bodies and diameter was recorded.

Biological yield (BY) and dry yield

Biological yield in g/500 g packet was recorded by weighing the whole cluster of fruiting body without removing the lower hard and dirty portion. Dry yield was recorded by weighing the fruiting bodies after drying.

Biological efficiency

Biological efficiency was calculated using the formula: [fresh weight of harvested mushrooms/dry matter content of the substrate] x 100.

Determination of moisture content

The moisture content of the compost was estimated by drying 2 g of fresh compost in an oven at 80°C for three consecutive days. It was cooled in a desiccator and weighed. The moisture content was calculated by the following formula.

Moisture content (%) = [(Fresh weight - dry weight) / fresh weight] x 100.

Statistical analysis

The experiment was done completely randomized design with three replications (n = 3). Data was analyzed and graph was constructed by Microsoft Excel.

RESULTS

Days to complete mycelium running

Days to complete mycelium running in mushroom bed ranged from 16 to 39 days on different agro-industrial waste (Figure 1). The lowest days to complete mycelium running was recorded on Wood chips control bed (16±1 days). The highest number of days required to complete

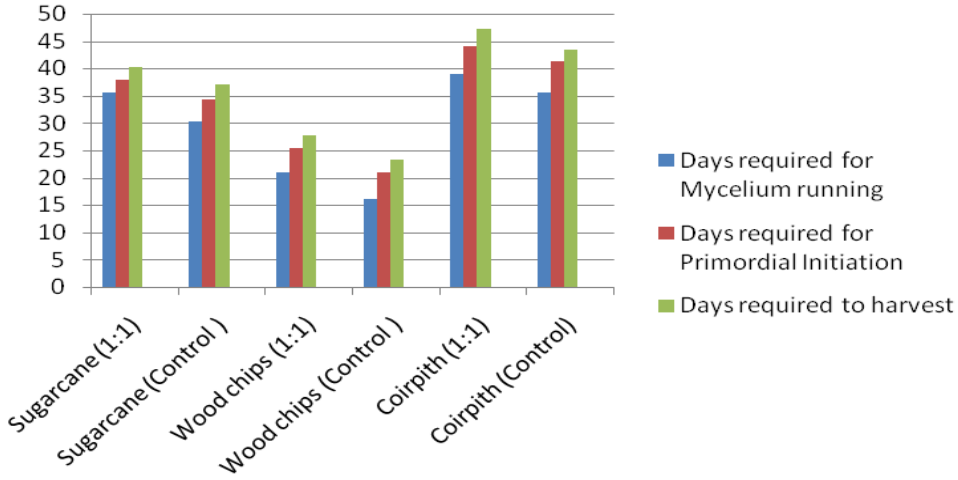

Figure 1. Time required for growth performance of *Pleurotus flabellatus*.

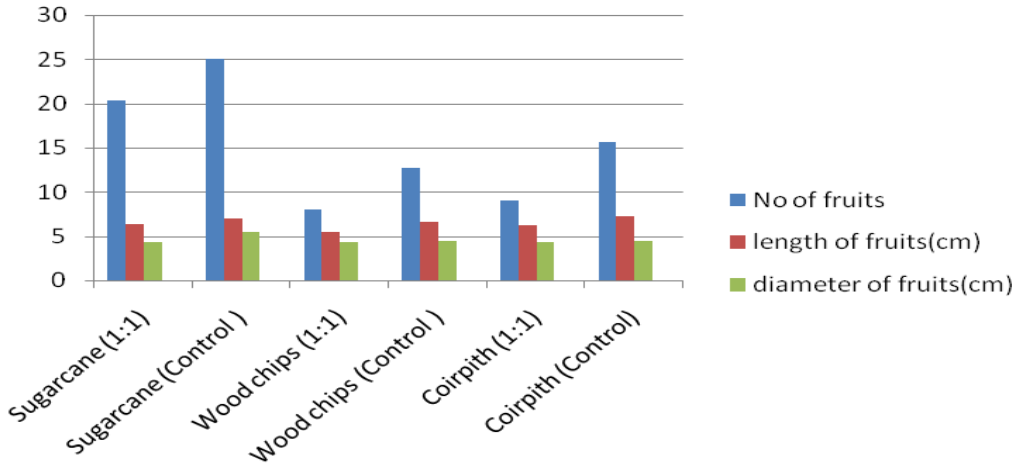

Figure 2. Effect of agro-industrial wastes on the development and size of fruiting bodies of *Pleurotus flabellatus.*

mycelium running was recorded with Coir pith: Cooked Fish Waste (1:1) bed (39±1 days).

Days required for primordial initiation and first harvest

Primordial initiation was minimum on wood chips control bed (21±1 days). The highest number of days required from opening to primordial initiation was on the coir pith : cooked fish waste (1:1) bed (44±1) followed by coir pith control bed (41.33±1.52). Days to first harvest ranged from 23.33±1.52 to 47.33±0.57 on different substrate. The maximum number of days required from opening to first harvest was recorded with coir pith: cooked fish waste (1:1) bed (47.33±0.57) (Figure 1).

Effective fruiting body per packet

Number of well-developed fruiting body was recorded and presented in Figure 2. Dry and pin headed fruiting body was discarded but twisted and tiny fruiting body was included during counting. The highest number of effective fruiting body was obtained from sugarcane control bed (25±2), while the lowest was obtained from control wood chips : cooked fish waste (1:1) bed (8±0.26).

Length of stalk and diameter of pileus

The highest length of stalk was recorded in coir pith control bed (7.23±0.20 cm) followed by sugarcane control bed (7.0±1 cm), wood chips control bed (6.66±0.32 cm),

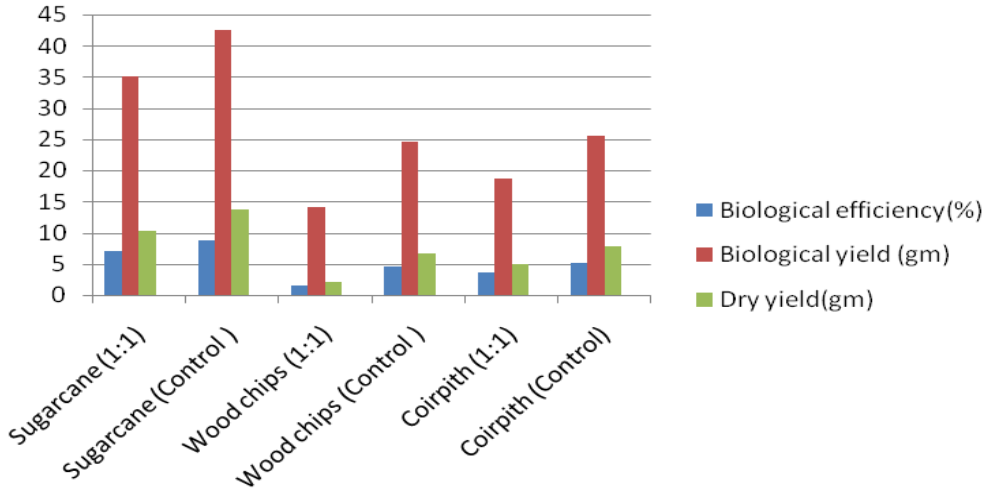

Figure 3. Effect of agro-industrial wastes on yield of *Pleurotus flabellatus*.

Figure 4. **(a)** Sugar cane: Cooked Fish Waste (1:1) bed, **(b)** coir pith: cooked fish waste (1:1) bed, **(c)** Woodchips: Cooked Fish Waste (1:1) bed.

sugarcane: cooked fish waste (1:1) (6.3±0.2 cm), coir pith: cooked fish waste (1:1) (6.26±0.20 cm), wood chips: cooked fish waste (1:1) bed (5.46±0.4 cm). The diameter of pileus is reported to increase the quality and yield of mushroom. The highest diameter of pileus was observed in mushroom grown on sugarcane control bed (5.43±0.15 cm), while the lowest value was recorded on coir pith: cooked fish waste (1:1) bed (4.26±0.15 cm) (Figure 2).

Biological efficiency (BE) and biological yield

The BE of oyster mushroom ranged from 4.87±0.02 to 11.63±0.02% on different substrates (Figure 3). The highest biological efficiency was recorded on control bag with sugarcane (11.63±0.02%) and the lowest BE was observed in wood chips: cooked fish waste (1:1) bed (4.87±0.02%). The maximum biological yield was recorded with control bag of Sugarcane (58.05±0.88 g) followed by sugarcane: cooked fish waste (1:1) (48.26±0.14 g) (Figure 4a), woodchips control (37.03±1.30 g), coir pith control (32.8±0.13 g), coir pith: cooked fish waste (1:1) (25.61±0.26 g) (Figure 4b). The lowest biological yield was observed in wood chips: cooked fish waste (1:1) (24.43±0.30 g) (Figure 4c).

Dry yield per packet

Dry yield of oyster mushroom grown on agro-industrial wastes varied from 4.24±0.11 to 19.33±0.11 g (Figure 3).

The highest dry yield was recorded on sugarcane control (19.33±0.11 g); the lowest dry yield was found on wood chips: cooked fish waste (1:1) (4.24±0.11 g).

DISCUSSION

Substrate is one of the important parameter in mushroom cultivation as mushrooms depend on substrates for nutrition to support mycelia growth and development into mushroom fruiting bodies. For the growth and penetration of the mycelium into basal substrates, which ultimately influences fruiting of mushrooms, the structure and porosity levels of substrate are important factors to be considered. (Mutemi Muthangya et al., 2014). In this study, days to complete mycelium running in mushroom bed ranged from 16 to 39 days on different agro-industrial waste, which was more or less similar 16-25 days of *Pleurotus ostreatus* reported by Shah et al. (2004) working on wheat straw, leaves and saw dust. Islam et al. (2009) also suggested that the total days required completing mycelium running in Mahogany, Jam, Shiris, Kadom, and Jackfruit sawdust was 25 days. While Mango and Coconut sawdust took 26 and 30 days respectively to complete mycelial running. On the other hand, Baysal et al. (2003) found the fastest spawn running (15.8 days) in waste paper as substrate. From the results obtained in this study, days to first harvest ranged from 23.33±1.52 to 47.33±0.57 on different substrate. The maximum number of days required from opening to first harvest was recorded with Coir pith: Cooked Fish Waste (1:1) bed (47.33±0.57). Similar results on *Pleurotus* sp. have been reported by Shah et al. (2004); Mutemi Muthangya et al. (2014). In this study, the biological yield was recorded with Coir pith: Cooked Fish Waste (1:1) bed (25.61±0.26g). In the present study the number of days required from opening to primordial initiation was on the Coir pith: Cooked Fish Waste (1:1) bed (44±1).The number of days required from opening to first harvest was recorded with Sugarcane: Cooked Fish Waste (1:1) bed (40.33±1.15). Tripathy et al. (2011); Mshandete (2008) observed that the duration of different growth stages of cultivated mushrooms are affected by several factors which would include, but not limited to, type of substrates and supplements used, the type of species and/or the strain employed, spawn type and the rate of inocula/spawn applied, spawning method, spawning /cropping containers as well as on the prevailing mushroom growing conditions. In this study, the dry yield of oyster mushroom grown on agro-industrial wastes varied from 4.24±0.11 to 19.33±0.11g. This result is similar to the findings of Ashrafuzzaman et al. (2009) who investigated the dry yield varied possibly due to the variation of chemical composition of different substrates. Recently, Lopez Castro et al. (2008) stated that *Pleurotus* waste was adequate to sustain the growth of *Salvia officinalis* by improving air porosity and mineral content of the soil. This research is to looking at the possible use of fish waste and agro-industrial wastes for mushroom cultivation, which would provide medicinal food and encourage the biological conversion processes of agro-industrial wastes. Composting fish waste is a relatively new, practical and an environmental sound alternative to disposing of fish waste. It is economical, fairly odorless and a biologically beneficial practice for seafood operations. The study has shown that composting is safe and a cost effective solution for the seafood industry.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Morphological, pathogenic and genetic variability in *Colletotrichum capsici* causing fruit rot of chilli in Tamil Nadu, India

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Chilli (*Capsicum annuum* L.) fruit rot disease caused by *Colletotrichum capsici* under tropical and subtropical conditions, results in qualitative and quantitative yield losses. Twenty (20) isolates of *C. capsici* were collected from conventional chilli growing areas of Tamil Nadu. In culture, most of the isolates produced cottony, fluffy or suppressed colonies. However, no significant differences were noticed in shape and size of conidia. The reaction of the 20 isolates on an indigenously developed differential set of *Capsicum* cultivars indicated the existence of different virulences in Tamil Nadu chilli populations. The genetic relationship between 20 morphological groups recognized within *C. capsici* was investigated using random amplified polymorphic DNA (RAPD) analysis. Molecular polymorphism generated by RAPD confirmed the variation in virulences of *C. capsici* and different isolates were grouped into two large clusters. The pathological and RAPD grouping of isolates suggested no correlation among the test isolates.

Key words: Chilli, *Colletotrichum capsici,* variability, RAPD.

INTRODUCTION

Chilli (*Capsicum annuum* L.) is an important spice crop of India. Chilli originated in the Latin American regions of New Mexico as a wild crop in and around 7500 BC. It is affected by several fungal, bacterial and viral diseases, of which chilli anthracnose causes considerable damage, inflicting severe quantitative and qualitative losses. The estimated loss due to this disease ranged from 8 to 60% in different parts of India (Suthin Raj et al., 2009). The fungus *Colletotrichum capsici* infects both unripe (green) and ripe (red) chilli fruits, and survives on seed as acervuli and microsclerotia (Suthin Raj et al., 2009). Infection of *C. capsici* will be higher in mature stage than in the early stage of chilli plant (Suthin Raj et al., 2013). The objective of this study was to investigate the variability in *C. capsici* populations infecting chillies by using morphological, pathological and molecular approaches.

MATERIALS AND METHODS

Isolation and identification of *C. capsici* isolates

Diseased chilli fruits showing typical symptom of fruit rot disease were collected fresh from 20 conventional chilli growing areas of

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Tamilnadu. The pathogens isolated from each of these localities formed one isolate of *C. capsici.* The pathogens were isolated on potato dextrose agar (PDA) medium from the diseased specimen showing typical symptoms. The infected portion of the fruit was cut into small bits, surface sterilized in 0.1% mercuric chloride solution for 30 s, washed in repeated changes of sterile distilled water and plated onto PDA medium. The plates were incubated at room temperature (28 ± 2°C) for five days and were observed for fungal growth. The fungus was purified by single spore isolation technique (Rangaswami, 1958). Identification of the isolate was confirmed by comparing it with the culture obtained from ITCC, IARI, New Delhi and the purified isolates were maintained on PDA slants.

Morphological characteristic of *C. capsici* isolates in Tamil Nadu

Measurements of 100 spores were taken under the microscope (Magnification 45 × 10x) by using ocular and stage micrometers. The mean values and the range were determined. Fine sliced pieces of potato tuber, ripe chilli fruit and green chilli fruit were boiled for 10 min and the extracts were filtered. To the extract, other ingredients of the medium were added and the volume was made up to 1000 ml with distilled water and autoclaved at 1.04 kg cm^{-2} for 15 min. The sterilized and warm medium was poured into sterilized Petri plates (90 mm) in 20 ml quantities and allowed to solidify. The isolates were inoculated at the centre of the plate by placing a seven-day-old 8 mm PDA culture disc of the pathogen. The plates were incubated at room temperature (28 ± 2°C) with four replications. The radial growth of the mycelium was measured eight days after inoculation. The colony colour and the growth pattern on the culture media were also recorded.

DNA extraction

The total genomic DNA of *C. capsici* was isolated from mycelia. Isolates were incubated at 28°C for four days in tubes containing 20 ml of potato dextrose broth, agitated at 180 rpm. Mycelia were harvested by filtration through filter paper, dried between two layers of filter paper and stored at -80°C for further use. Dried mycelium was ground to fine powder with pestle and mortar using liquid nitrogen and transferred to 1.5 ml Eppendorf tube. 600 µl cetyltrimetylammonium bromide (CTAB) was added and incubated at 65°C for 30 min, tubes were vortexed every 10 min. After cooling at room temperature, equal volume (600 µl) of chloroform: isoamyl alcohol (24:1, v/v) was added in fume hood cabinet, gently mixed for 20 to 30 min and centrifuged at 7000 rpm for 5 min at 4°C. The aqueous phase was transferred to new tubes and repeat CIA extraction. After the second CIA wash, the DNA was precipitated by adding 300 µl isopropanol, tubes were gently mixed and incubated at room temperature for 30 min. Tubes were centrifuged at 12000 rpm for 10 min and supernatant was decanted. The DNA pellet was dissolved in 50 µl of ddH_2O.

RAPD

Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) procedure described by Williams et al. (1990) was used by the following of a reaction mixture of 25 µl volume which consisting of 2.5 µl of 10X PCR buffer, 2.5 µl of 25 mM $MgCl_2$, 0.5 µl dNTPs mix (10 mM each of dATP, dCTP, dGTP, dtp), 0.4 µl Taq DNA polymerase, 2.0 µl of primer, 1.0 µl of genomic DNA and 16.1 µl of sterilized double-distilled water. The reaction mixture was vortexed and centrifuged at 12000 rpm for 2 min. Amplification was carried out in a thermal cycle by using three temperature profiles, programmed for initial DNA denaturation at 94°C for 3 min, followed by 35 cycles consisting of DNA denaturation for 30 s at 94°C, primer annealing at 35°C for 30 s and polymerization for 1 min at 72°C with a final extension period of 10 min at 72°C. Amplification products were separated on 1.5% agarose gel in 1X TAE buffer at 110 V for about 3 h; 100 bp + 1 kb DNA ladder mix (Fermentas) (0.5 µg/µl) was run for weight size comparison. Gels were stained with ethidium bromide for 30 min; they were visualized with UV light and photographed.

Analysis of DNA fingerprints

Differences in fingerprinting patterns between isolates were assessed visually. Polymorphisms including faint bands that could be scored unequivocally were included in the analyses. Presumed homologous bands were scored as present (1) or absent (0) to create a binary matrix. A similarity matrix was generated from the binary data using DICE similarities coefficient in SIMQUAL program of NTSYS-PC Package (Rohlf, 2000). Cluster analysis was done with the Unweighed Pair Group Method with Arithmetic Mean (UPGMA) in the SAHN program of NTSYS-PC Package and a dendrogram was constructed based on genetic distances.

RESULTS

Chilli fruits showing typical fruit rot and leaf spots symptoms were collected from 20 conventional chilli growing areas of Tamil Nadu with a view to find out the pathogenic fungi involved in leaves and fruit rot of chilli in the different places (Suthin Raj and Christopher, 2009; Suthin Raj et al., 2012). From the samples of fruit and leaves, the pathogen was isolated and purified. Identification of the isolates were confirmed by comparing with the culture obtained from ITCC, IARI, New Delhi and the purified isolates named as Cc1 to Cc20 were furnished. Similar results on pathogenic and cultural properties were identified by Singh et al. (2010).

The isolates of *C. capsici* exhibited variation in respect of colony colour and growth pattern. The isolates of Cc1, Cc2, Cc3 and Cc11, which produced acervuli in a scattered manner, had fluffy and raised colonies. The isolate Cc1 produced various colours in different media. The colony colours of the different isolates of *C. capsici* in the various media are furnished in Table 1. Udhayakumar and Usha rani (2010) attributed the growth of fungus to nutritional factors. Minnatallah and Kumar (2005) also reported similar results. Twenty (20) isolates of *C. capsici* were isolated from chilli plants in different parts of Tamil Nadu. Initially, 20 random 10-mer primers (Genei, Bangalore) were screened to select primer exhibiting maximum polymorphism, of these six primers (Table 2) which produced easily scorable and consistent banding patterns were used for RAPD analysis of test isolates and consistent producing 4 to 8 bands of 0.3 to 2.5 Kb and the dendrogram drawn from the RAPD patterns using unweighed pair group method with arithmetic mean (UPGMA). The RAPD pattern obtained with primer code S1027 base sequence ACGAGCATGG is shown in Figure 1. Of the 20 races that displayed intra-race variability for RAPD phenotype, only four isolates (Cc-5, Cc-31

Table 1. Characteristics of twenty isolates of *C. capsici* in Tamil Nadu, India.

Isolate	Colony colour				
	Potato dextrose agar	**Czapek's dox agar**	**Richard's agar**	**Green chilli extract agar**	**Ripe chilli fruit extract agar**
Cc1	White	Greyish white	Greyish white	Blackish white	White
Cc2	Black	Black	Greyish white	Black	Black
Cc3	White	White	White	White	Black
Cc4	White	Greyish white	Greyish white	Black	Black
Cc5	Black	White	Whitish black	Whitish black	White
Cc6	Black	White	Greyish white	Black	White
Cc7	Black	Black	White	Greyish black	Brownish black
Cc8	Black	White	Whitish black	Greyish white	White
Cc9	White	White	White	White	White
Cc10	White	White	White	Black	Brownish black
Cc11	White	Black	Black	Black	White
Cc12	Greyish black	Whitish black	Greyish black	White	White
Cc13	Whitish black	Black	Whitish black	White	Black
Cc14	Whitish black	White	White	White	Greyish white
Cc15	White	White	Greyish black	Black	White
Cc16	White	Black	White	Whitish black	Black
Cc17	Greyish black	Black	Greyish black	Greyish white	Black
Cc18	White	Black	Greyish black	Black	White
Cc19	White	White	White	White	White
Cc20	Black	White	White	White	White

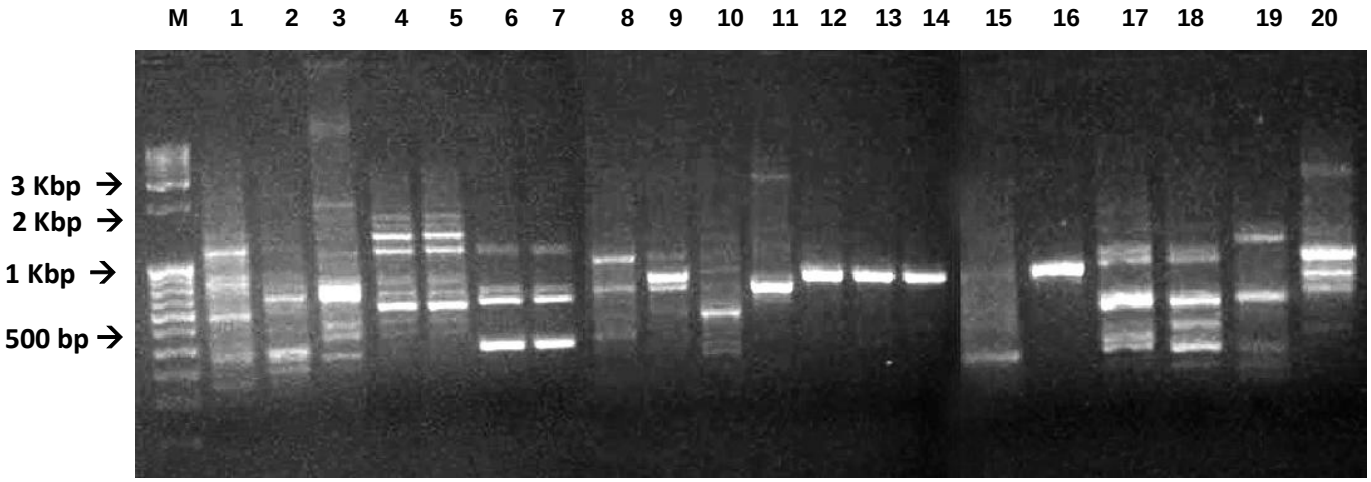

Figure 1. Random amplified polymorphic DNA profile observed in *C. capsici* isolates using S1027 primer from fruit rot of chilli in Tamil Nadu, India. Lane M: 100 bp to 1 Kbp DNA Ladder. Lanes 1- 20: *C. capsici* isolates.

and Cc-27, Cc-35) showed 89% similarity.

According to the dendrogram (Figure 2), *C. capsici* isolates in Tamil Nadu can be divided into two main groups. The first group includes one isolate which was isolated from Sivapuri. The second group contains other three isolates of *C. capsici* which were isolated from Virurdhunagar, Naduthittu and Sattur respectively. There was no congruence between the RAPD and virulence pattern of test isolates.

DISCUSSION

Different species of *Colletotrichum* were reported to possess a high degree of molecular variability when evaluated by RAPD analysis. RAPD markers were used

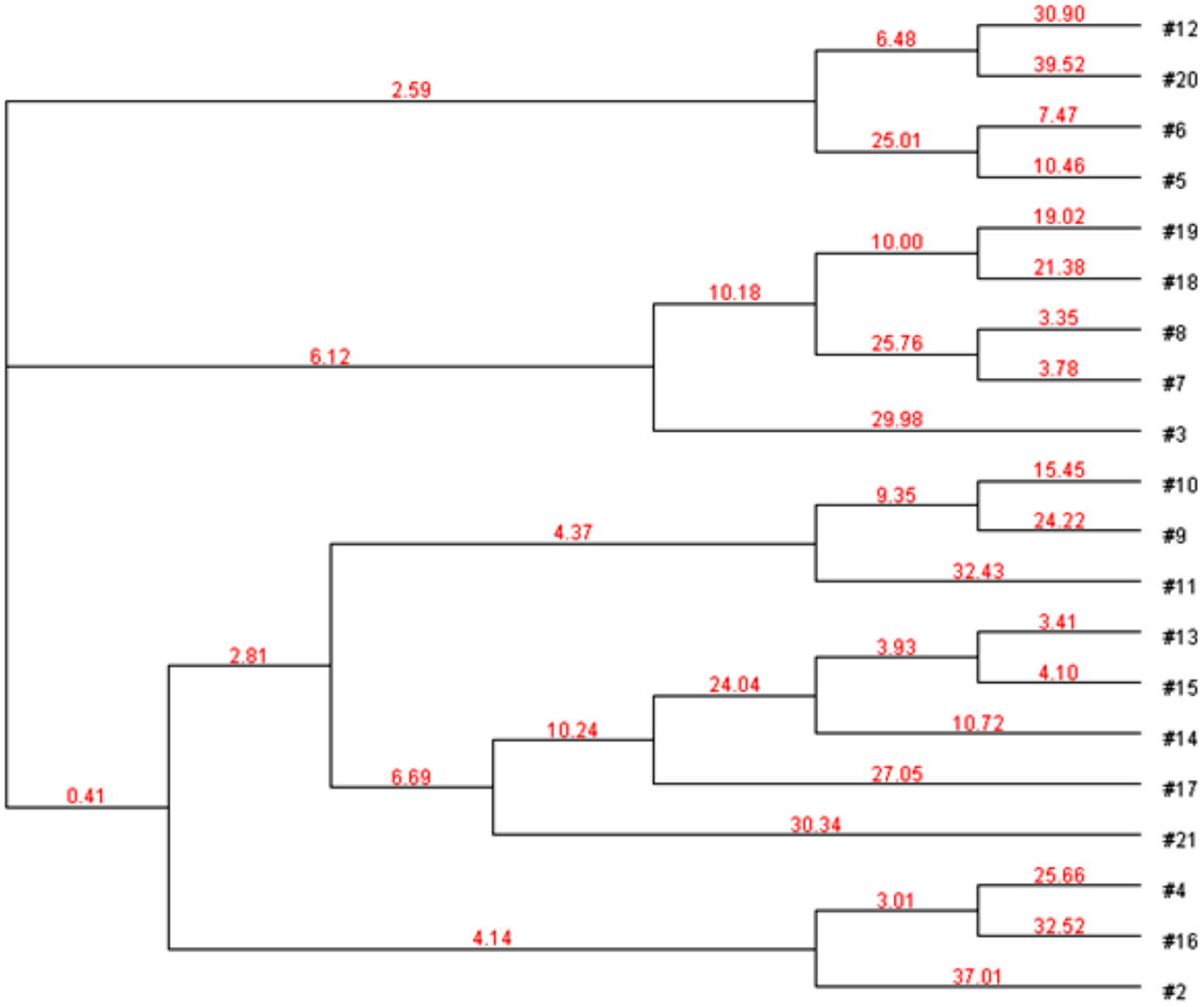

Figure 2. UPGMA Dendrogram of twenty (20) *C. capsici* isolates from chilli fruit rot in Tamil Nadu, India.

Table 2. Nucleotide sequence of primers generating amplification of *C. capsici*.

Primer code	Base sequence (5'-3')
S1027	ACGAGCATGG
S1063	GGTCCTACCA
S1089	CAGCGAGTAG
S1136	GTGTCGAGTC
S1155	GAAGGCTCCC
S1181	GACGGCTATC

for the intraspecific characterization of a number of pathogens (Balardin et al., 1997). This approach was found useful for proper identification of races of *Colletotrichum lindemuthianum* as it yielded race-specific amplified DNA profiles (Balardin et al., 1997). Singh et al. (2010) categorized 51 isolates of *Colletotrichum gloeosporioides* into four groups following RAPD analysis, which were previously categorized on the basis of morphology and virulence. They did not find any correlation between classification of different isolates by RAPD and rate of growth of isolates in culture or their geographic origin. On the basis of polymorphism, Sharma et al. (2010) reported similar results on rhizospheric *Trichoderma* isolates.

The colony colours of the different isolates of *C. capsici* in the various media are furnished. Udhayakumar and Usha rani (2010) attributed the growth of fungus to nutriational factors. Minnatallah and Kumar (2005) also reported similar results. In each RAPD group, isolates from different locations were present indicating high genotypic diversity. Variability among the isolates of *Ustilago*

segetum revealed a high genetic variability by RAPD as shown by Padmaja et al. (2006). Isolates that were identical for virulence were most often dissimilar for RAPD markers. Isolates classified in race group CCP-I was distributed across different areas. Similar variation in response of tomato types were also reported by Hema et al. (2007).

Conclusion

It is concluded that *C. capsici* causing fruit rot or anthracnose of chillies in Tamil Nadu possessed variable populations as is evident from differential inoculation and RAPD analysis. The phylogenetic grouping based on RAPD data did not appear to be congruent with morphological and virulence pattern. RAPD-based DNA fingerprinting could be one of the methods of studying genetic diversity in *C. capsici* in the absence of a definite differential set. The use of a large number of random primers or use of other DNA markers may yield race-specific DNA marker for the detection of particular race.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Management of foliar and soilborne pathogens of cowpea (*Vigna unguiculata* L. Walp) with two garlic varieties (*Allium Sativum* A. Linn)

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White and pink garlic extracts were tested for their antifungal potentials on mycelial radial growth, spores and sclerotial production of *Macrophomina phaseolina* (Tassi) Goid, *Colletotrichum destructivum* O gara and *Colletotrichum capsici* (Syd) Butler and Bisby pathogens of cowpea *in vitro*. Water or ethanol extracts of common pink and common white garlic varieties were tested at a concentration of 250 ppm while sterile distilled water served as control. *In vivo* study was based on white garlic extract alone at 0, 50 and 100% concentrations on *M. phaseolina* and *C. capsici.* Data were subjected to ANOVA and means was separated at P=0.05. Water extracted white garlic gave over 90% inhibitions of mycelial growth of *M. phaseolina* and gave higher inhibitions than water or ethanolic pink garlic extracts on all the three pathogens. It is not significantly lower P=0.05 than conventional fungicide benomyl at 0.5 gai/kg. However, *in vivo* result was phytotoxic to cowpea seeds at 100% concentration of white garlic extract. On cowpea variety TVx 3236, *C. capsici* inoculated seed germination and pathogen control was 100%, also, *M. phaseolina* inoculated seeds germination was 100% but there was no pathogen control at 50% garlic extract. In contrast, cowpea variety IT84S-2246-4 seed germination was 77% when treated with *M. phaseolina* and 100% with *C. capsici* but reduced pathogen control at 50% garlic extract.

Key words: Pink and white garlic, fungal pathogens, conventional fungicide, cowpea varieties, germination, phytotoxicity, control.

INTRODUCTION

The use of synthetic agro-chemicals in developing countries to reduce pests and diseases present serious environmental problems to growers especially peasant farmers who depend on agriculture for sustenance and livelihood. Most often, the response of increasing demand for food has always been in form of increased use of synthetic agro-chemicals. These chemical pesticides are often poorly handled, resulting in the contamination of the environment. Globally, there is increasing demand in favor of natural pesticides among consumers. Peasant farmers may

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also find this approach more acceptable due to the fact that it ease the part of accessibility in their local environment and it flow freely with their normal way of life. Nature herself has offered us a profusion of plants for use in natural crop protection for a cleaner and safer environment. One of such plants is *Allium sativum* (garlic). This plant is widely grown in Nigeria and it is believed to be antimicrobial and antifilarial. Traditionally, *A. sativum* has been used in the treatment of worms and dysentery in children and adults. In the kitchen, garlic is used for seasoning. There are several varieties of garlic but the most popular are the common white and the common pink (Ngeze, 2001).

Cowpea (*Vigna unguiculata* (L). Walp) is a legume of preference in the tropic where it supplies millions of peoples as their means of livelihood as food, fodder and cash. Disease is one of the important constraints to maximum production. All stages of growth as well as different parts of the plant is attacked by pests and diseases. Important foliar fungal pathogens of cowpea in Nigeria are anthracnose and brown blotch caused by *Colletotrichum destructivum* and *Colletotrichum capsici*. (Emechebe and Shoyinka, 1985). Ashy stem and charcoal rot diseases occurred sporadically on cowpea and are caused by seed and soil-borne *Macrophomina phaseolina* (Tassi) Goid, a pathogen of variable host (Emechebe and Shoyinka, 1985). Its ability to colonize several hosts and sporadic occurrence makes it more threatened. Over 500 hosts had been reported for *M. phaseolina* while loss from soybean field alone was estimated to be 77% in the USA (Hewitt, 1988). Although, benzimidazole based compounds are available against these pathogens; risk of fungicide resistance, the scale of economy and wrong attitude of consumer to chemical handling makes them less practicable for peasant farmers. Alternative methods that are cheaper, environmental friendly, biodegradable, easy to prepare and apply are desirable. Hence, the major objective of this study is to explore the use of garlic extracts in the control of these pathogens.

MATERIALS AND METHODS

Fresh garlic was purchased from an open market in Ibadan, Oyo state, Nigeria. The garlic lobes were washed and air-dried on the laboratory bench. 25 g each of the common white and common pink garlic were weighed separately and blended for 4 min with commercial warring blender and made up to 250 ml with sterile distilled water. The suspension was transferred into a beaker and wrapped up with aluminium foil. It was steam-boiled at 60°C for 2 h to enhance the extraction of the active components. The resulting solution was decanted and filtered with Whatman® filter paper No. 1. Sterilization of the water extract was obtained by passing the solution through 0.45 µm millipore filters. Ethanol extraction was carried out using the same method but was concentrated to about 2 ml with rotary evaporator and left to air-dry so as to remove any traces of ethanol. 250 mL of sterile distilled water was added to re-dissolve the concentrated extract, which served as the stock. Sterile distilled water was used as control.

Isolation of the test pathogens

The *M. phaseolina* fungal isolates used were isolated from germinating cowpea variety IT84S-2244-6 grown on infected soil. The two species of *Colletotrichum* (*C. destructivum* O gara*, C. capsici* (Syd) Butler and Bisby used for these studies were isolated from IITA cowpea demonstration field, IITA Ibadan, Nigeria. Blotter method as described by Waller et al. (1998) was employed during the study.

Fungitoxicity test *in vitro*

Antifungal potential of the extracts were tested on these pathogens using acidified potato dextrose agar (APDA). One milliliter (1 ml) of each extract was dispensed into 9 cm - diameter Petri dish. 3 cm mycelia disc taken from the margin of 7 days old cultures of test pathogens viz.- *C. destructivum C. capsici* and *M. phaseolina* was placed at the centre of each Petri dish containing the extracts and molten APDA. The plates were incubated at 28 to 30°C for 7 days. Fungitoxic effects (fungicidal and fungistatic) were observed daily, beginning at day 2 until day 7 after inoculation and incubation. Benomyl, a conventional fungicide at 0.5 g ai/litre of water and sterile distilled water served as positive and negative control, which was set up without the plant extracts. Colony diameter was measured after 7 days as the mean growth along two axes on two predrawn perpendicular lines on the reverse side of the plate. Percentage fungitoxicity were expressed according to Awuah (1989).

$$MP = [(M1 - M2) / M1] \times 100$$

Where: MP = percentage inhibition of mycelial growth, M1 = Mycelial growth in control Petri dish without extract or fungicide, M2 = Mycelial growth in extract/fungicide Petri dish.

Control of artificially inoculated cowpea seed with garlic extract

Approximately 300 g seeds of cowpea variety TVx 3236 and IT84S-2246-4 each was surfaced sterilized with 0.5% NaCl for 1 min, air-dried in the laminar flow and treated with garlic extract by soaking the seed in the extract for 3 mins at 50 and 100% concentrations. Cowpea seeds were later inoculated separately with the pathogens at 4.0×10^6 conidia/ml of *C. capsici*. The inoculum meal of *M. phaseolina* was used to coat cowpea seeds completely by scraping aseptically the surface of a fully grown (5 day old) mycelial mat of *M. phaseolina*, marched together and two drops of Tween 80® was added to enhance adhesiveness to the treated seeds. The cowpea seed were planted on three seeds per pot to a depth of 3 cm in a heat-sterilized soil. Observations on damping-off symptom (pre- and post- emergence), seedling growth and disease severity were observed for 21 days after inoculation and planting. A control treatment was set up with sterile distilled water.

Statistical analyses

All experiments were repeated twice. Data were analyzed using generalized linear model (GLM) procedure of statistical analysis software (SAS, Institute 2001). Means comparison were carried out at $P = 0.05$.

Table 1. Inhibition of mycelia radial growth (%) of three fungal isolates treated with pink or white garlic extracts and compared with benomyl.

Pathogen	Pink garlic 250 ppm at 7DAI		White garlic 250 ppm at 7DAI		Conventional fungicide
	Water	Ethanol	Water	Ethanol	Benomyl 0.5 gai/Kg
M. phaseolina	72.0[a]	65.1[a]	91.5[a]	82.6[a]	90.0[a]
C. destructivum	36.0[b]	34.2[b]	41.5[b]	33.39[b]	100.0[a]
C. capsici	22.0[c]	27.7[c]	54.1[a]	34.45[b]	100.0[a]

DAI= Days after inoculation; Means followed by the same letter within a column are not significantly different P=0.05.

RESULTS

At day 4 after inoculation, the *in vitro* result gave 100% inhibition of the mycelial radial growth, conidial and sclerotial germination of the three pathogens treated with 250 ppm of both pink and white garlic extracts. However, at 5 days after inoculation, pink or white garlic extracted in ethanol generally gave reduced inhibition relative to garlic extracted with water till seven days after inoculation. Petri dishes treated with white garlic extracts and inoculated with *M. phaseolina* gave highest inhibition percentages of 91.5 and 82.6% in water and ethanol garlic extracts, respectively, relative to the control plate at concentration of 250 ppm (Table 1). Similarly, Petri dishes treated with common pink garlic and inoculated with *M phaseolina* showed reduction of mycelial growth of 72.0 and 65.1% respectively on water and ethanol pink garlic extract after 7 days of inoculation. The least percentage inhibition of 27.7% was observed on *C. capsici* when treated with the common pink garlic while the white variety showed its least inhibition percentage of 34.45% on *C. destructivum* (Table 1). This *in-vitro* result on *M. phaseolina* was not significantly different at P=0.05 and compared favourably with conventional fungicide benomyl at 0.5 gai/kg (recommended concentration rate). Hence, only white garlic extract was tested in the *in-vivo* study.

There were general reductions in germination and plant stands of cowpea varieties when treated at 100% concentration of white garlic extract at 7 and 21 days after sowing (DAS) compare with 50% and untreated control. This is an indication of phytotoxicity of the extracts at 100% concentration on both cowpea varieties. However, cowpea variety TVx 3236 at 50% concentration of garlic extract gave complete seed germination (100%) when artificially inoculated with *M. phaseolina* and higher plant stand than cowpea variety IT84S-2246-4 at 50% concentration (Table 2). Seedling damping-off incidence, severity and control of both pathogens were greater at 50% garlic extract concentration at 7DAS in the greenhouse. However, the efficacy of the extracts reduced with days on cowpea variety IT84S-2246-4, such that by 21DAS control experiments gave higher cowpea stands than cowpea stands treated with 50% garlic extract concentration. On the other hand, germination, final stand and control of seedling damping –off caused by *C. capsici* was complete (100%) on cowpea variety TVx 3236 when treated with 50% garlic extract concentration. One hundred percent concentration was however not good for the control of damping –off diseases caused by the two pathogens and on either of the two cowpea varieties (Table 2).

DISCUSSION

Garlic extracts effectively inhibited the mycelial radial growth, conidial and sclerotial germination of the three pathogens *in vitro*. This observation is in agreement with the work of Sangoyomi (2004) who reported that aqueous extract of garlic effectively inhibited mycelia growth, conidia, pycnidia and sclerotial production of *Butryodiplodia theobromae, Aspergillus niger, Sclerotium rolfsii, Rhizoctonia solani,* and *Nattrassia mangifera,* fungal pathogens of yam in storage. Islam al. (2001) reported the control of C*olletotrichum* sp. and *M. phaseolina* in jute using garlic extract at ratio 1:2. Recent studies from Perelló et al. (2013); Baraka et al. (2011) also supported efficacy of garlic extract in control of wheat pathogens and/ or its oil extract at 100 and 500 ppm against root rot fungal pathogens of date palm. Allicin found in garlic are highly volatile (Freeman and Kodera, 1995) and readily membrane permeable. Slusarenko et al. (2008) described the constituents of damaged garlic tissues as volatile antimicrobial substance allicin (diallylthiosulphinate) and the substrate alliin (S-allyl-L-cysteine sulphoxide) which mixes with the enzyme alliin-lyase. Allicin also undergoes thiol-disulphide exchange reactions with free thiol groups in proteins. This mode of action is suggested as the basis for its antimicrobial activities. In their studies, garlic juice was effective against some ranges of bacteria, fungal and oomycetes.

Water or ethanol garlic extract inhibited the growth of fungal pathogens in this study and it conforms to previous studies by Onyeagba et al. (2004); Tagoe et al. (2009).

This study also found that while both water and ethanol extracting solvents are effective solvent for extraction of antimicrobial constituent from garlic, garlic water extract gave higher control than its ethanol counterpart. Although

Table 2. Effect of concentrations of garlic extracts on germination and control of seedling damping-off of two cowpea varieties.

Pathogens	Garlic concentration	Cowpea varieties			
		TVx 3236		IT84S-2242-4	
		Germination %		Germination %	
M. phaseolina		**7DAS**	**21DAS**	**7DAS**	**21DAS**
	0	66.67	61.11	66.67	66.67
	50	100	55.56	77	16.67
	100	0	0	0	0
	Mean	55.56	38.89	47.89	27.78
	SE	29.40	19.51	24.13	20.03
C.capsici		**7DAS**	**21DAS**	**7DAS**	**21DAS**
	0	55.55	22.23	55.26	33.33
	50	100	100	100	0
	100	0	0	0	0
	Mean	51.83	40.74	18.46	11.11
	SE	28.93	30.32	18.42	11.11

Where DAS = Days after sowing;

not significantly different (P = 0.05), but the ease of extraction and reduced cost of producing potential biofungicides from garlic was demonstrated in this study. Studies on garlic extracts by Rasul et al. (2012) corroborated this finding. They reported that garlic water extract was superior and resulted in better extraction yield than ethanol water or methanol water garlic extracts. The fact that white garlic extract consistently proved more effective at inhibiting the mycelial growth of the three pathogens than the pink garlic, is an indication of variation in active constituents due to the existing garlic varieties. Most information on antifungal potential of garlic had not distinguished garlic potencies by their existing varieties. Climatic variation significantly affects color, shape and variety of garlic grown in a particular area (www.seeds.ca/proj/gcgc/). However, this study suggests that garlic varieties grown within the same climatic environment (tropical environment) exhibit antifungal variations on some cowpea pathogens due to their varieties. Germination of cowpea seedlings also varied at different concentrations and between the varieties tested. Permeability and tolerance level of cowpea variety to extracts may be responsible for such response. Permeability to water, oxygen and radicle protrusion properties of seed coat were positively correlated with seed coat color due to phenolic compound present in different species (Debeaujon et al., 2000). Zamin and Ajmal (2010), found that water uptake pattern of seed coat is dependent on exposure to saline or non-saline conditions as well as seed variety. Singh et al. (1980) also observed that neem oil extract inhibited the germination of *Cicer arietimum* gram seed at high concentration. As suggested by Ekpo (1999), pre-determination of extract concentration prior to application would bring out the expected benefits of natural fungicides.

Control of *M. phaseolina* on cowpea variety TVx3236 at 50% concentration showed gradual reduction from 100 to 55.56% at 7 and 21DAS, respectively, compared with 77.0 and 16.67% on cowpea variety IT84S-2246-4. This result suggests instability of garlic extract in controlling the pathogens with days. Rasul et al. (2012), reported decrease in pH as well as increase acidity of garlic water extract with time. Hence, application of garlic extract on seedlings along with seed treatment is suggested. According to Freeman and Kodera (1995) garlic extract easily loses its stability. Total control (100%) of *C. capsici* was observed on cowpea variety TVx3236 at 7 and 21 DAS. However, on cowpea variety IT84S-2246-4 control at 7DAS was 100% but there was no control at 21DAS. Adebitan et al. (1992) reported the resistance to *C. capsici* by cowpea variety TVx 3236 in the field. These studies therefore suggest that application of 50% garlic extracts concentration on the cowpea pathogens was more suitable as natural fungicides. It also showed that garlic extracts can be used to control the pathogens if applied at appropriate concentrations and can serve as cost reduction and environmentally friendly alternative to synthetic fungicide against seedling damping-off of cowpea caused by *M. phaseolina* and *C. capsici in-vivo.* It is important to note that high inoculum level employed under

this study is not always obtained in nature. Therefore, the study may have under-estimated the potency of garlic extract under field condition. Area of further research will be to assess the effect of garlic extract under field conditions at different agro-ecological zones. Also, improvement on phytotoxicity by working on the volatility of garlic extract will enhance antimicrobial benefits of garlic extract on other cowpea varieties. It is believed that cheap and readily available natural fungicide can be obtained from this type of work.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

α-L-Arabinofuranosidase from *Penicillium janczewskii*: Production with brewer's spent grain and orange waste

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The highest production of α-L-arabinofuranosidase by *Penicillium janczewskii* in medium with brewer's spent grain and orange waste was observed when cultivation was carried out in pH 5.0 at 25°C, for 8.5 days under shaking. The α-L-arabinofuranosidase present in the crude filtrate was optimally active at 60°C and pH 4.0; it was stable in a wide range of pH, maintaining 90% of the activity from 2.0 to 8.0. The enzyme was very stable at 40°C, maintaining 90% of the activity within 1 h. The estimated half-life at 50°C was 10 min, and at 60 and 70°C, it was 5 min. This enzyme was activated in the presence of Ba^{2+}, Ca^{2+}, Mn^{2+} and NH_4^+ while the ions Pb^{2+}, Mg^{2+}, Hg^{2+}, Co^{2+} and Cu^{2+}, as well as PMSF, DTT, β-mercaptoethanol, EDTA and SDS inhibited it.

Key words: α-L-Arabinofuranosidase, *Penicillium janczewskii*, enzyme production, enzyme properties, industrial wastes.

INTRODUCTION

Arabinosyl residues are largely distributed in the side chains of some hemicelluloses, especially arabinoxylans. Usually, the presence of side chains can restrict the full enzymatic hydrolysis of hemicelluloses, preventing the complete degradation of these polymers (Numan and Bhosle, 2006; Peng et al., 2012).

Due to xylans heterogeneity and complexity, an enzymatic complex is required for their complete hydrolysis. The xylanolytic enzymes act cooperatively to degrade not only the main chain, but also the side chains of the polymer; this second group of enzymes is known as accessory or auxiliary enzymes (Polizeli et al., 2005). Among them, α-L-arabinofuranosidases (EC 3.2.1.55) are responsible to hydrolyze non-reducing ends of α-L-1,2-, α-L-1,3- and α-L-1,5-arabinofuranosyl residues of many branched oligo- and polysaccharides, especially those containing large amounts of arabinose, as arabinoxylans (found in cereal grain and softwood), arabinan and other polysaccharides containing L-arabinose (Saha, 2000; Numan and Bhosle, 2006; Seiboth and Metz, 2011).

The production of α-L-Arabinofuranosidases (ABF) can be observed in several microorganisms, as bacteria and fungi, and also in some plants (Lagaert et al., 2014). The production of theses enzymes by microorganisms is strongly influenced by the composition of the culture medium, mainly in relation to the carbon source. Agricultural and agro-industrial residues present potential to be used as raw material in the production of fuels and

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Abbreviations: ABF, α-L-arabinofuranosidase(s); **BSG,** brewer's spent grain; **OW,** orange waste.

chemical compounds in large scale, and their utilization to compose culture medium for microbial growth is a strategy both economical and environmentally interesting since these sources are usually available in large quantities at low cost.

Many fungi have been studied for the production of xylanolytic enzymes, and some *Penicillium* species are considered good ABF producers (Chávez et al., 2006). Among them, the production of this enzyme has been previously related in Penicillium sp. (Lee et al., 2011), *P. capsulatum* (Filho et al., 1996), *P. canescens* (Sinitsyna et al., 2003), *P. purpurogenum* (de Ioannes et al., 2000), *P. brasilianum* (Panagiotou et al., 2006), *P. funiculosum* (Guais et al., 2010) and *P. chrysogenum* ((Sakamoto and Thibault, 2001; Sakamoto and Kawasaki, 2003; Sakamoto et al., 2011).

The use of ABF individually or in addition to other hemicellulases represents a promising tool in biotechnological processes as in the production of medicines, for example, anti-glycemic and anti carcinogenic compounds, in the improvement of wine flavor, pre-treatment of cellulosic pulps, clarification of juices, production of animal feed with improved quality, bioethanol production, among others (Saha, 2000; Numan and Bhosle, 2006).

A *Penicillium janczewskii* Zaleski strain, isolated from soil of Brazilian Rainforest (São Paulo, Brazil) was characterized as a good xylanase producer (Tauk-Tornisielo et al., 2005), motivating studies about its xylanolytic system. A previous work about the production of xylanase, β-xylosidase and ABF showed that brewer's spent grain (BSG) and orange waste (OW) are good substrates for ABF production (Terrasan et al., 2010) by this fungal strain.

Some new knowledge about the biochemical properties of ABF is essential for the future application of the enzyme, of the xylanolytic complex or even of the fungus in biotechnological processes. Thus, the aim of this study was to evaluate the main conditions for optimized ABF production by *Penicillium janczweskii* in liquid medium with brewer's spent grain and orange waste, and also to study some biochemical properties of this extracellular crude enzyme.

MATERIALS AND METHODS

Microorganism

P. janczewskii was deposited in the Environmental Studies Center Collection, CEA/UNESP, São Paulo State, Brazil. It was maintained on Vogel solid medium (Vogel, 1956) with 1.5% (w/v) wheat bran at 4°C, and cultured periodically. The cultures were inoculated in the same medium with 1.5% (w/v) glucose and incubated for conidia production during 7 days at 28°C.

Growth conditions for enzyme production

Liquid cultures were prepared in Vogel medium supplemented with 2% (w/v) mixture of BSG and OW (1:1, w/w). Erlenmeyers flasks (125 ml) containing 25 ml of the medium were inoculated with one milliliter of a 1.10^7 conidia ml^{-1} suspension and incubated initially at 28°C for seven days under static conditions.

Effect of temperature and pH on enzyme production

The effect of temperature on enzyme production was verified by culturing the fungus at 20, 25, 30 and 35°C. The pH of the medium was adjusted to 6.5 and cultivation was carried out for seven days under static conditions. The effect of pH was verified by adjusting the pH of the medium to values ranging from 3.0 to 8.0, with 0.1 M HCl or NaOH. Cultivation was carried out at 25°C for seven days under static conditions.

Time-course of *P. janczewskii* growth and α-L-arabinofuranosidase production

Time-course of *P. janczewskii* growth and ABF production was carried out under static and under shaking (120 rpm) conditions pH 5.0 at 25°C. Fungal growth was estimated indirectly by the determination of intracellular protein content.

Preparation of crude enzyme

The crude filtrate was separated by vacuum filtration with Whatman No. 541 and used for extracellular enzyme activity and protein determinations. The mycelium was disrupted with sand and suspended in McIlvaine buffer pH 4.0; after centrifugation (9000×*g*, 20 min.), this supernatant was used for intracellular protein determination.

Enzyme and protein determinations

ABF activity was determined in a buffered reaction mixture containing 0.25% (w/v) *p*-nitrophenyl-α-L-arabinofuranoside (Sigma) and appropriately diluted enzyme solution. Initially, the reactions were carried out in McIlvaine buffer pH 4.0 at 50°C. The reaction was stopped by the addition of a saturated sodium tetraborate solution and the absorbance was measured at 405 nm (Kersters-Hilderson et al., 1982). One unit of activity was defined as the amount of enzyme required to release 1 µmol of *p*-nitrophenol per min in the assay conditions. Specific activity was expressed as enzyme units per milligram of protein. The protein concentration was determined as described by Lowry et al. (1951), using bovine serum albumin as standard. Data represent the average and deviation of triplicates.

Biochemical characterization of crude α-L-arabinofuranosidase from *P. janczewskii*

Determination of optimum pH and temperature

Optimum pH was determined by assaying enzyme activity in pH ranging from 2.0 to 8.0 with different buffers, that is, 0.05 M Gly-HCl buffer for pH 2.0, 2.5 and 3.0, and McIlvaine buffer from pH 3.0 to 8.0 at 50°C. The optimum temperature was determined by assaying enzyme activity in temperatures from 30 to 90°C, with 5°C intervals, in McIlvaine buffer pH 4.0.

Stability in different pH and temperature

The stability in different pH was verified by measuring residual activity after incubation of the crude enzyme in different buffers in the pH range from 2.0 to 9.0 for 24 h at 4°C. The crude extract was

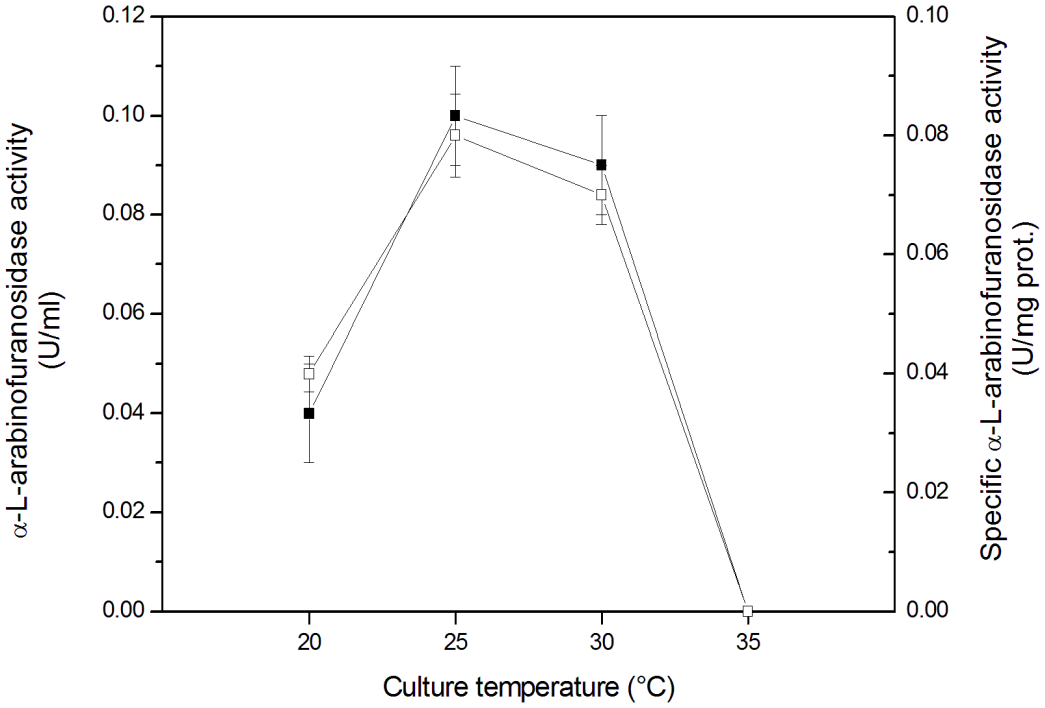

Figure 1. α-L-Arabinofuranosidase production by *P. janczewskii* in different temperatures. Growth conditions: Vogel liquid medium with brewer's spent grain and orange waste, pH 6.5, 7 days of cultivation under static conditions. ■, α-L-Arabinofuranosidase activity (U/ml); □, specific α-L-arabinofuranosidase activity (U/mg prot.).

incubated in 0.05 M Gly-HCl buffer for pH 2.0, 2.5 and 3.0, McIlvaine buffer from pH 3.0 to 8.0 and 0.05 M Tris-HCl buffer from pH 8.0 to 9.0. Thermal stability was evaluated by residual activity determination after incubation of the crude enzyme at 40, 50, 60 and 70°C for different periods. The half-life (T_{50}) of the enzyme was estimated for each temperature.

Influence of ions and other substances

The following substances were added to the reaction medium $CuCl_2$, $ZnSO_4$, $MnSO_4$, $BaCl_2$, $CaCl_2$, NH_4Cl, NaCl, $Pb(CH_3COO)_2$, $MgSO_4$, $CoCl_2$, $HgCl_2$, sodium citrate, SDS, EDTA, PMSF, DTT and β-mercaptoethanol, at final concentrations of 2 and 10 mM. The results were expressed in relation to the control (without any substance).

RESULTS AND DISCUSSION

Optimization of the α-L-arabinofuranosidase production by *P. janczewskii*

When *P. janczewskii* was cultivated in different temperatures (Figure 1), the highest ABF production was verified at 25°C (0.10 U/mL), intermediate values were observed at 20 and 30°C (0.04 and 0.09 U/mL, respectively), and no activity was verified at 35°C. The specific activity presented the same profile with the highest value observed at 25°C (0.08 U/mg prot.). The production of ABF by *P. brasilianum* and *P. purpurogenum* was also observed in intermediate temperatures (27-28°C). The enzymatic production by *P. brasilianum* also decreased at temperatures higher than 36°C (de Ioannes et al., 2000; Panagiotou et al., 2007). Some other *Penicillium* presented optimum temperature for ABF production slightly more elevated, at 30°C, such as *P. capsulatum*, *P. chrysogenum* and *P. funiculosum* (Filho et al., 1996; Guais et al., 2010; Sakamoto and Thibault, 2001).

In cultures carried out in different pH (Figure 2), the highest enzyme production was observed in cultures carried out at pH 5.0 (0.18 U/ml). At pH 4.0 and 6.0 cultures, the activity levels were also elevated (0.16 e 0.11 U/ml, respectively). The activity decreased at pH 3.0 and 7.0 (0.01 U/ml), and at pH 8.0, no activity was detected. The specific activity showed the same profile with highest value observed at pH 5.0 (0.13 U/mg prot.). The results are similar to others ABF from different species of *Penicillium*. The highest ABF production by *P. capsulatum* and *P. chrysogenum* was verified at pH 4.5 and 5.0, respectively (Filho et al., 1996; Sakamoto and Kawasaki, 2003); for both *P. brasilianum* and *P. funiculosum* it was observed at pH 6.0 (Panagiotou et al., 2006; Guais et al., 2010).

The time-course in static culture (Figure 3a) showed that enzyme production increased from the third to the sixth day (0.14 U/ml) of cultivation, in which was observed the peak of ABF production, and activity remained

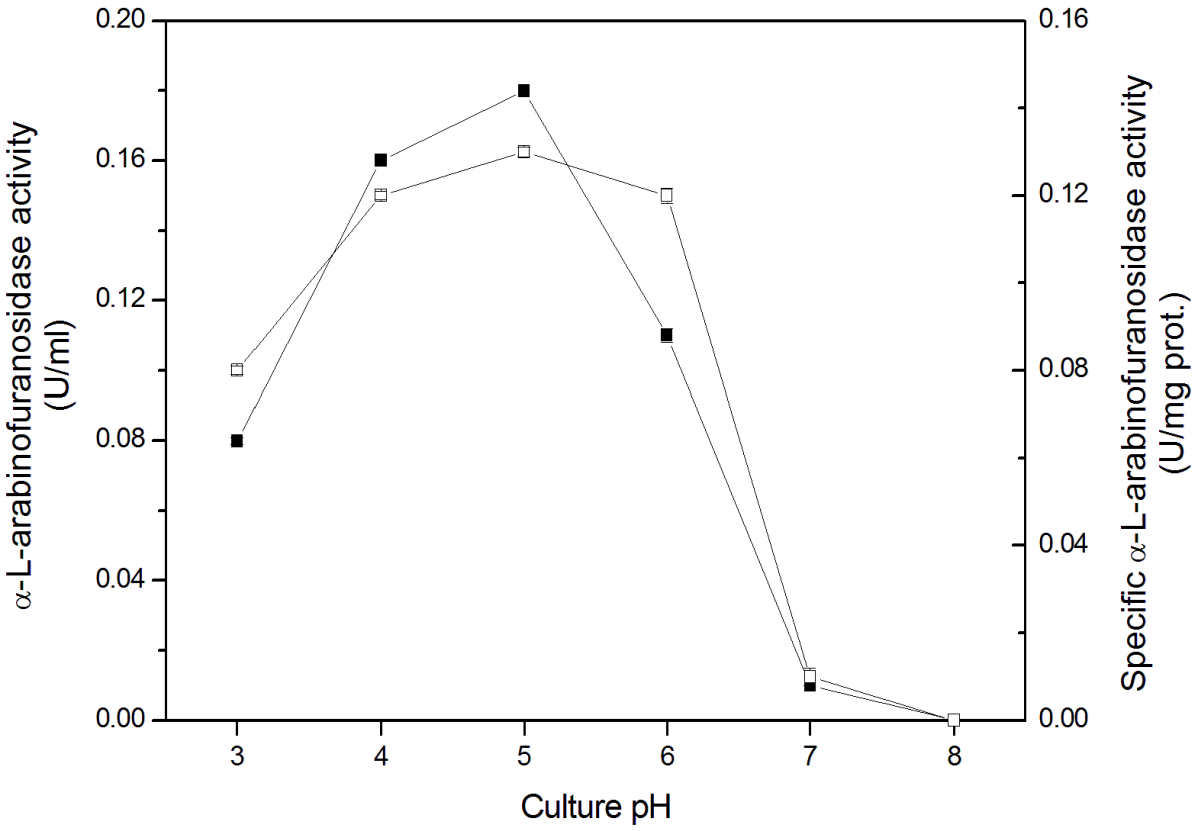

Figure 2. α-L-Arabinofuranosidase production by *P. janczewskii* in different pH. Growth conditions: Vogel liquid medium with brewer's spent grain and orange waste, at 25°C, for seven days under static conditions. ■, α-L-arabinofuranosidase activity (U/ml); □, specific α-L-arabinofuranosidase activity (U/mg prot.).

unchanged over the following days of cultivation. The specific activity presented similar profile but the highest value was observed at the seventh day (0.12 U/mg prot.) decreasing slightly until the tenth day (0.11 U/mg prot.).

In shaking condition (Figure 3b), the production increased from 3.5 days of cultivation and the peak of activity was observed at the tenth day (0.7 U/ml). At 8.5, 9.0 and 9.5 days-old cultures high activity levels were also observed, corresponding to 0.66, 0.64 and 0.62 U/ml respectively. The specific activities in this case were different after the eighth day, and the values at the seventh and tenth day were very similar (0.57 and 0.55 U/mg prot., respectively). A *Penicillium* strain (Rahman et al., 2003) and *Talaromyces thermophilus* (Guerfali et al., 2010) showed elevated ABF production around the seventh (0.5 U/ml) and sixth days (0.26 U/ml) of cultivation, respectively, when grown under shake condition.

The ABF production by *P. janczewskii* under shaking was much higher than that observed under static conditions. Some *Penicillium* species as *P. chrysogenum* and *P. brasilianum* (Panagiotou et al., 2006; Sakamoto and Thibault, 2001) present elevated ABF production in static condition while for *P. purpurogenum* and *P. funiculosum* was observed under agitation (de Ioannes et al., 2000; Guais et al., 2010).

The cultivation of *P. janczewskii* in static condition presented maximum growth (1.5 mg prot.) on the sixth day while under shake, the peak of fungal growth (4.17 mg prot.) was verified earlier at 3.5 day of cultivation (Figure 4). During the stationary growth phase, in static condition, it was observed that the filamentous mycelium covered the entire surface of the medium, while under agitation the mycelium formed pellets that were not immersed in the medium (not shown). Usually, static condition results in growth of a heterogenic filamentous mycelium, in which the superficial layers are in continuous contact with oxygen but with little interaction with the medium while the lower layers, are constantly in contact with the medium but with reduced oxygen availability. Depending on the intrinsic physiological characteristics of each microorganism, this situation can result in growth inhibition or in elevated production of secondary metabolites. Under agitation, a more homogeneous system can be achieved favoring the formation of mycelium pellets that in some cases is a prerequisite for the successful production of secondary metabolites or enzymes (Braun and Vecht-Lifshitz, 1991). This second situation apparently was the case of *P. janczewskii* in relation to ABF production.

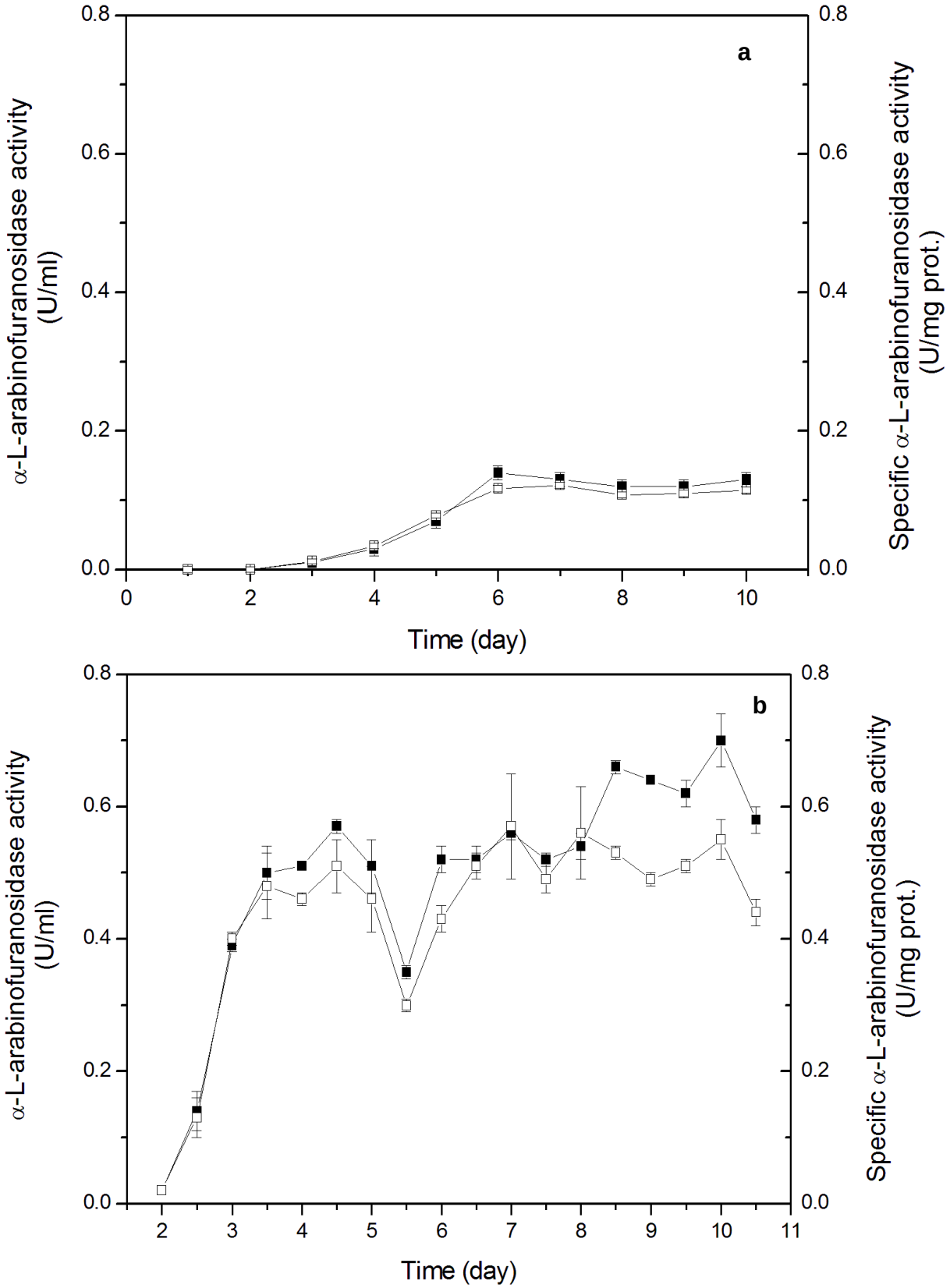

Figure 3. Time-course of α-L-arabinofuranosidase production by *P. janczewskii* in static **(a)** and shaking (120 rpm) **(b)** conditions. Growth conditions: Vogel liquid with medium brewer's spent grain and orange waste, pH 5.0, 25°C. ■, α-L-Arabinofuranosidase activity (U/ml); □, specific α-L-arabinofuranosidase activity (U/mg prot.).

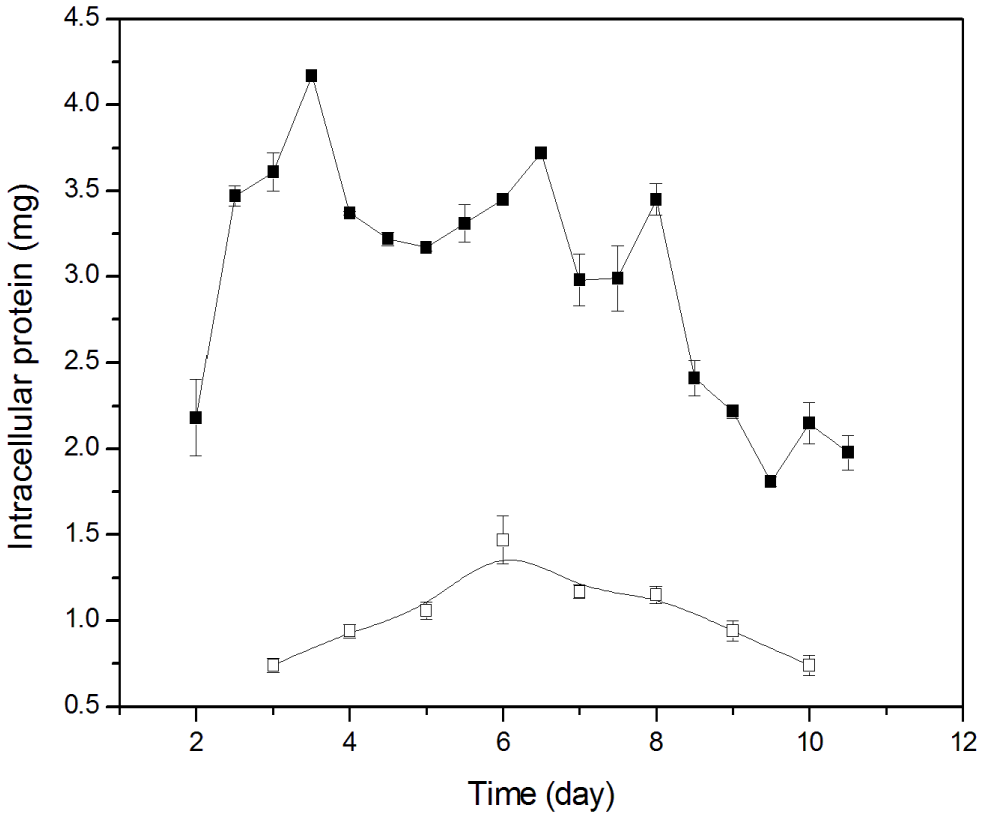

Figure 4. Time-course of *P. janczewskii* growing in static and shaking (120 rpm) conditions. Growth conditions: Vogel liquid medium with brewer's spent grain and orange waste, pH 5.0, 25°C. ■, Intracellular protein in shaking condition (mg); □, intracellular protein in static condition (mg).

Biochemical properties of α-L-arabinofuranosidase from *P. janczeswskii*

The ABF showed optimum activity in pH 4.0; in pH 3.5, the activity was similar to this and in pH 4.5, the activity was approximately 80% of the maximum observed. In pH 5.0, the activity strongly decreased since only 20% of the maximum was observed, and from this pH, the activity gradually decreased (Figure 5). Fungal ABF usually present optimum activity at acid pH as verified for those from *P. capsulatum*, *P. purpurogenum* and *P. chrysogenum* that also present optimum activity at pH around 4.0 (Filho et al., 1996; de Ioannes et al., 2000; Sakamoto and Kawasaki, 2003).

The optimum temperature was observed at 60°C; at 55°C, it was also elevated corresponding to 90% of the maximum activity, at 50 and 65°C, it decreased to 60% of the maximum. In other temperatures, the activity remained below 50% of the maximum and at 90°C almost no activity was observed (Figure 6). The ABF produced by several other mesophilic *Penicillium* strains usually present optimum activity in temperatures between 50 and 60°C. The ABF from Penicillium sp, *P. purpurogenum*, *P. chrysogenum* and *P. brasilianum* are optimally active at 50°C (Lee et al., 2011; Panagiotou et al., 2006; de Ioannes et al., 2000; Sakamoto and Kawasaki, 2003) and those from *P. capsulatum* and *P. funiculosum* at 60°C (Filho et al., 1996; Guais et al., 2010), as well as the one from *P. janczewskii*. Exceptionally, the optimum temperature for the *P. canescens* ABF is 70°C (Sinitsyna et al., 2003). The activity at elevated temperature is important for an application of this enzyme in processes that are carried in this condition, for example the biobleaching in pulp and paper industries in which pulp is present at high temperature, approximately 60°C (Beg et al., 2001).

The enzyme was stable over a wide pH range maintaining more than 90% of the activity from pH 2.0 to 9.0, but at pH 10.0, only 22% of the activity was observed (Figure 7). Usually, fungal ABF exhibit a wide range of pH stability, for example, *P. brasilianum* ABF maintains more than 80% of the activity at the pH range from 3.0 to 11.0 after incubation at 10°C for 6 h (Panagiotou et al., 2006). The ABF from *P. chrysogenum* retains more than 80% of the activity at a pH range from 3.0 to 8.0 after incubation at 30°C for 16 h (Sakamoto and Thibault, 2001). The wide range of pH stability of the *P. janczewskii* ABF is very interesting since it allows the enzyme to be applied in industrial processes carried out at various pH values.

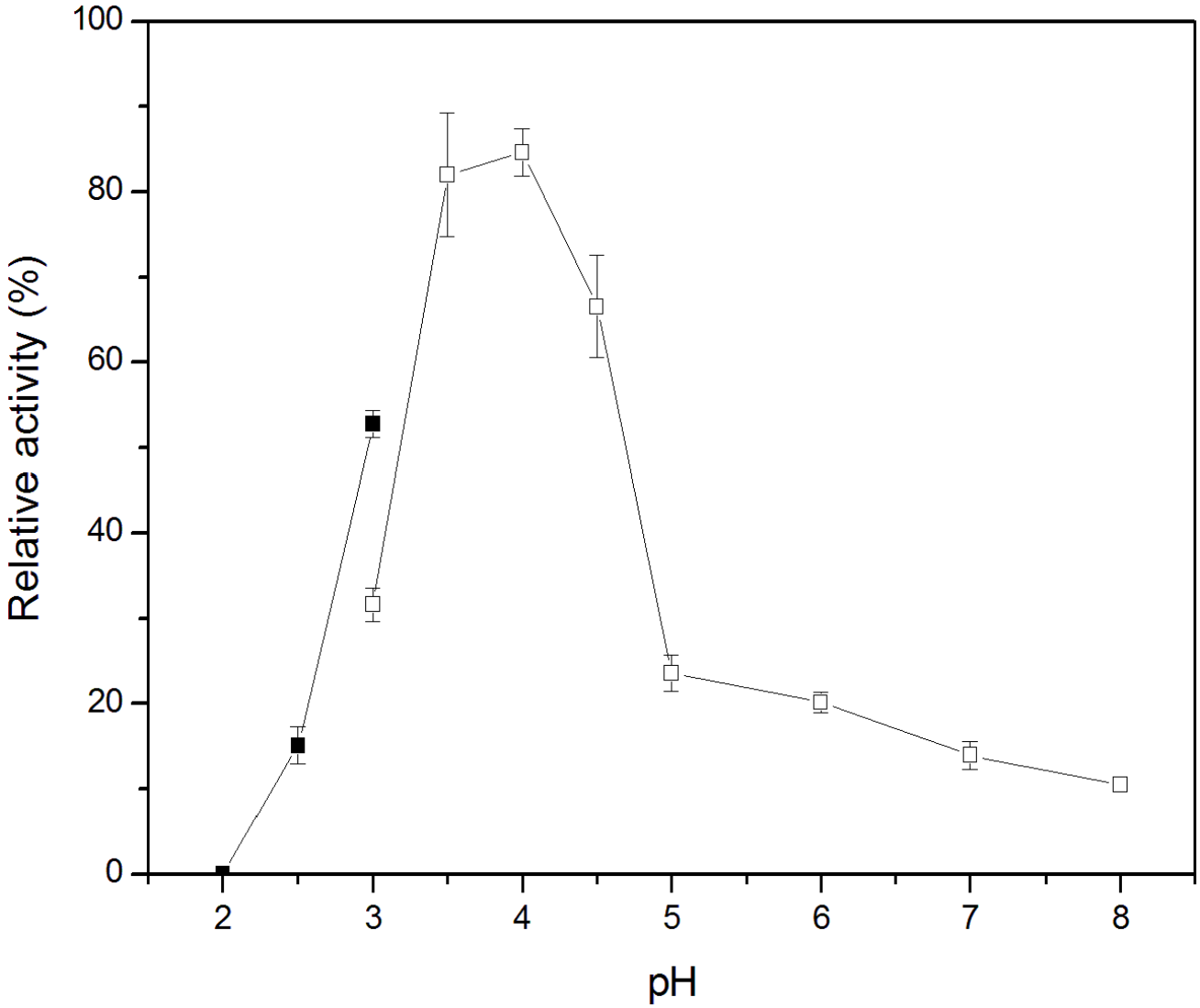

Figure 5. Influence of pH on α-L-arabinofuranosidase from *P. janczewskii*. Enzyme activity was assayed in 0.05 M Gly-HCl buffer (■) and McIlvaine buffer (□), at 50 °C.

The highest thermal stability of the enzyme was observed at 40°C maintaining more than 90% of the activity over a 1 h-period. At 50°C, the activity decreased to 52% of the initial after 10 min of incubation. The half-life values at 60 and 70°C were lower than 5 min (Figure 8). The purified ABF from *P. capsulatum* (Ara I) is very stable at 60°C and presents a half-life of 17.5 min at 70°C, while a second purified ABF from this fungus (Ara II) is less thermostable presenting a half-life of 8 min at 60°C (Filho et al., 1996). The *P. chrysogenum* ABF is not stable at 60°C; while at 50°C, more than 80% of the activity is retained after incubation for 1 h (Sakamoto and Kawasaki, 2003).

When the activity was assayed in the presence of different ions and substances (Table 1), the enzyme was slightly activated by Ba^{2+}, Ca^{2+}, Mn^{2+} and NH_4^+. On the other hand, Pb^{2+}, Mg^{2+} and Hg^{2+} negatively affected the enzyme, as well as Cu^{2+}, Zn^{2+} and Co^{2+} only at 10 mM. Mercury is known to interact with sulfhydryl groups of proteins changing and reducing its performance during catalysis. Similarly, the ions Cu^{2+} and Hg^{2+} also negatively affected the *P. chrysogenum* ABF while the other ions have no effect on the enzyme activity (Sakamoto and Thibault, 2001).

The activity was reduced by PMSF. This substance binds to serines and cysteines and it is commonly used to identify the presence of these amino acids in the active site of the enzymes, especially proteases. Usually, it is used to inhibit serine and cysteine proteases that can degrade the protein of interest after cell lysis (James, 1978). Thus, its negative effect on the ABF indicates the presence of these amino acids which should be important for catalysis mechanism and/or three-dimensional structure of this protein.

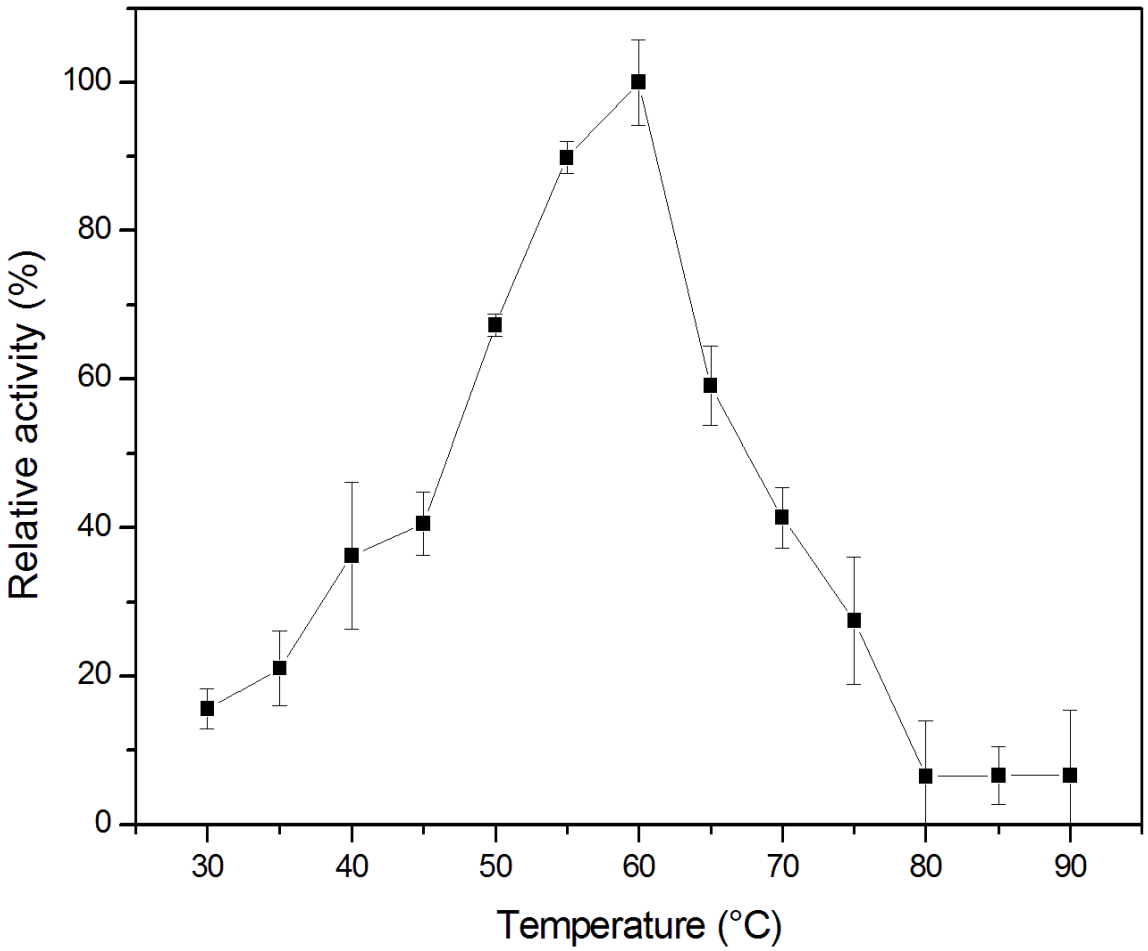

Figure 6. Effect of temperature on α-L-arabinofuranosidase from *P. janczewskii*., Enzyme activity assayed in McIlvaine buffer pH 4.0.

In different intensities, DTT and β-mercaptoethanol partially decreased the enzyme activity. These substances reduce disulfide bounds of proteins, and both are commonly used to prevent oxidation of sulfhydryl groups maintaining the enzyme activity. In this case, the decrease in the activity by these substances suggests that disulfide bridges should be important for maintaining the tertiary structure of the enzyme. This negative effect indicates indicate that ABF does not have cysteine but cystine, once it prevents S-H bounds of cysteine to be since these compounds promote breakage of disulfide bonds.

The addition of EDTA resulted in reduction of the enzyme activity suggesting that it requires a metallic ion as cofactor. The presence of SDS in both concentrations almost ceased the enzyme activity, indicating the importance of hydrophobic interactions for maintaining the three-dimensional structure of the protein.

Conclusions

P. janczewskii presented elevated production of α-L-arabinofuranosidase using brewer's spent grain and orange waste as substrates. Both residues are available in large scale and at low cost turning their use really interesting for industrial application. The selected culture conditions to increase the enzyme production were pH 6.5, at 25°C, for 8.5 days, being agitation (120 rpm) essential for elevated enzyme production by this fungal strain. This enzyme showed several properties interesting for future application in some industrial processes, since the optimum pH and temperature are 4.0 and 60°C, respectively. In the bleaching paper industry, for instance, the procedures are performed at temperatures higher than 50°C and pH values varying from 4.0 to 6.0. Besides, this enzyme presents great potential to be applied in biotechnological processes with other xylanolytic enzymes.

Conflict of Interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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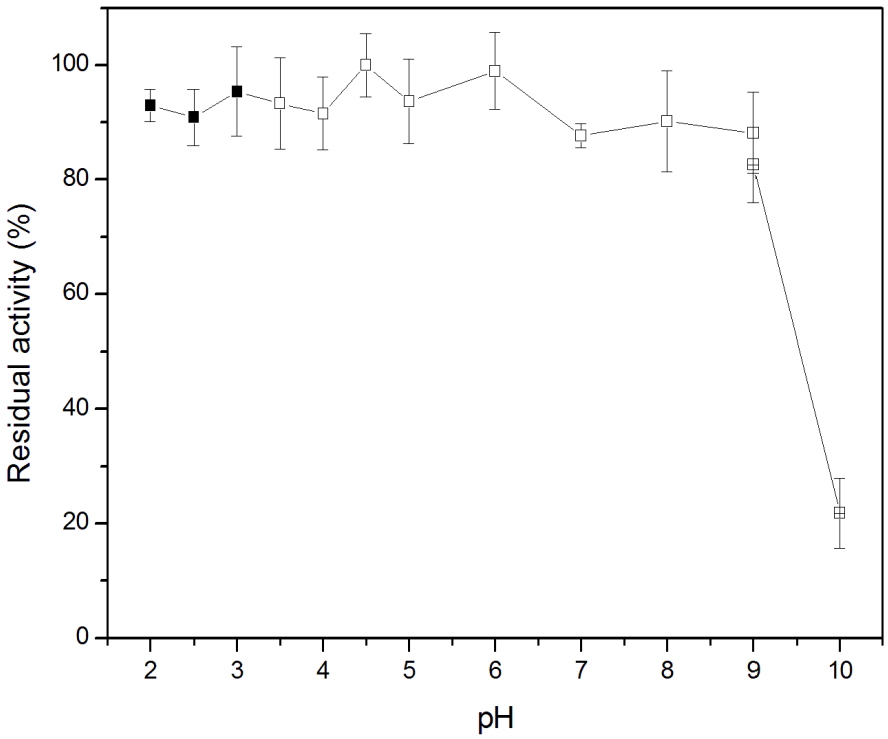

Figure 7. Stability in different pH of α-L-arabinofuranosidase from *P. janczewskii*. The residual activity was assayed in McIlvaine buffer pH 4.0 at 60°C after incubation in 0.05 M Gly-HCl (■), McIlvaine (□) and 0.05 M Tris-HCl (☒) buffers.

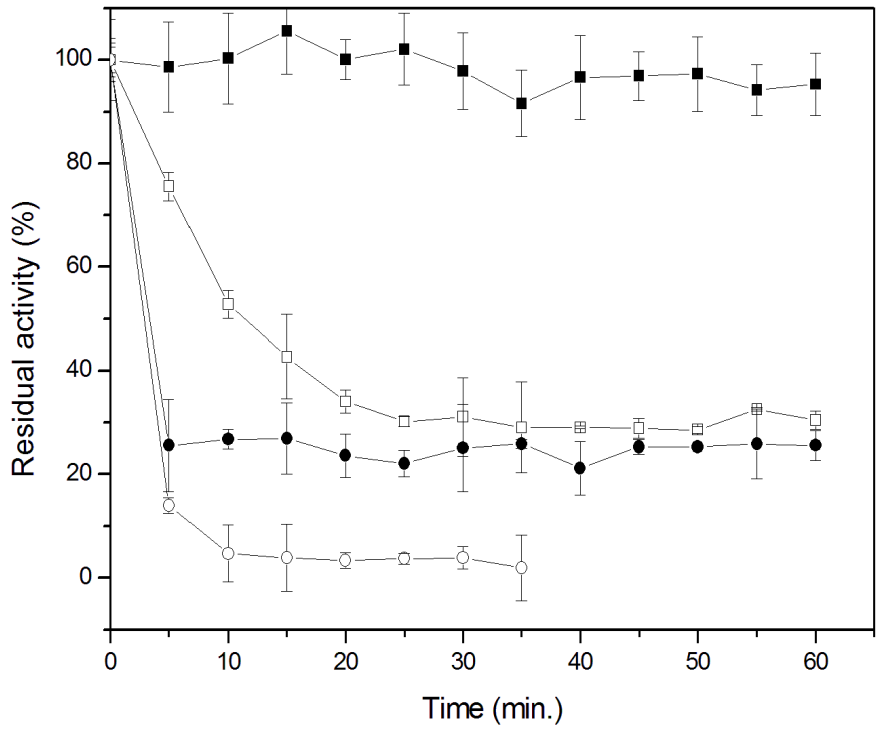

Figure 8. Thermal stability of α-L-arabinofuranosidase from *P. janczewskii*. Residual activity after incubation at 40 (■), 50 (□), 60 (●) and 70°C (○) assayed in McIlvaine buffer pH 4.0 at 60°C.

Table 1. Influence of ions and other substances on the α-L-arabinofuranosidase from *P. janczewskii*.

Substance	Relative Activity (%)	
	2 mM	10 mM
Control	100.0	100.0
$CuCl_2$	82.0 ± 0.4	8.2 ± 2.4
$ZnSO_4$	102.8 ± 3.0	77.0 ± 0.6
$MnSO_4$	111.5 ± 0.6	130.6 ± 11.6
$BaCl_2$	124.4 ± 1.1	112.5 ± 4.6
$CaCl_2$	120.6 ± 1.5	116.5 ± 3.7
NH_4Cl	114.4 ± 4.4	121.5 ± 5.0
NaCl	111.2 ± 5.3	110.8 ± 3.8
$Pb(CH_3COO)_2$	69.1 ± 4.5	47.7 ± 2.5
$MgSO_4$	61.8 ± 4.8	56.8 ± 11.2
$CoCl_2$	96.6 ± 0.2	33.7 ± 2.3
$HgCl_2$	44.6 ± 12.7	14.3 ± 21.9
Sodium Citrate	96.2 ± 0.5	106.7 ± 0.4
SDS	8.3 ± 16.7	5.1 ± 5.0
EDTA	68.5 ± 2.7	64.7 ± 2.2
PMSF	50.3 ± 0.6	22.0 ± 3.2
DTT	71.2 ± 3.6	62.5 ± 4.9
β-mercaptoethanol	57.2 ± 1.9	66.8 ± 2.6

The activity was assayed in McIlvaine buffer pH 4.0 at 60°C. Data are mean and standard deviation of triplicates.

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Full Length Research Paper

Nutritional composition of five food trees species products used in human diet during food shortage period in Burkina Faso

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The proximate compositions, minerals and amino acids contents of *Detarium microcarpum, Gardenia erubescens, Parkia biglobosa, Adansonia digitata* pulp, seeds of *Boscia senegalensis* and leaves of *A. digitata* were studied and quantified in Burkina Faso. Using the conventional procedures described by the Association of Official Analytical Chemists, proximate analysis was done. The minerals were determined by spectrometry and photometry. The profile and total amount of amino acids were determined by reverse phase HPLC using Pico-Tag method. The results showed that the protein content were ranged from 1.10 ± 0.20 to 24.23 ± 0.15%, total carbohydrate varied from 6.7 ± 0.11 to 79.73 ± 1.69%, lipids varied from 1.11 ± 0.08 to 4.65 ± 0.12% and ash from 1.16 ± 0.1 to 11.76 ± 0.10%. These results allowed distinguishing species which are potential sources of protein, calories and micronutrients. Consumption of these tree products can help to overcome nutrients deficiency that is prevalent in poor urban and rural areas of Burkina Faso.

Key words: Food composition, nutrition, food insecure, wild trees, Burkina Faso.

INTRODUCTION

Fruits and leaves from natural forest trees constitute a good food supply for many people during food shortage in Africa (Cook et al., 2000; Kim et al., 1997; Ayessou et al., 2009) and particularly in Burkina Faso

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(Bognounou, 1978; Bergeret and Ribot, 1990; Lamien et al., 1996; Helmfrid, 1998; Thiombiano et al., 2012). In several African countries, natural tree products constitute an important part of human diets and are also an important source of income (Falconer, 1990; Shackleton et al., 1998; Ayessou et al., 2009; Lamien et al., 2009). They are excellent source of minerals and vitamins; and also contain carbohydrates in form of soluble sugars, cellulose and starch (Nahar et al., 1990; Ayessou et al., 2009; Lamien et al., 2009). Mainly crudely consumed, they improve the daily food ration as an energy source and through their content in micronutrients (Parkouda et al., 2007; Ayessou et al., 2009; Kouyaté et al., 2009). Indeed in Africa, they constitute the most affordable and dietary sources of vitamins, trace elements and other bioactive compounds. Thereby, they form common ingredients in a variety of traditional native dishes for the rural population in developing countries (Humphrey et al., 1993). Consumption of indigenous trees products is among strategies to supplement diets (Falconer, 1990; Ayessou et al., 2009; Thiombiano et al., 2012). Several tree products which play pivotal role exist but undervalued and are seasonal (Ambé, 2001; Ayessou et al., 2009). Studies identified some of these food tree species (Arbonnier, 2004; Lamien et al., 2009; Thiombiano et al., 2012) and initiated studies of their nutritional potential (Glew et al., 1997; Parkouda et al., 2007; Ayessou et al., 2009; Kouyaté et al., 2009; Compaoré et al., 2011). However, most of these products have received little research attention, particularly their nutritional compositions. Their contribution to local diets is less understood and quantified. Therefore, the study was done to determine the proximate composition, minerals and amino acids content of five top wild trees products consumed during food shortage by rural population in Burkina Faso.

MATERIALS AND METHODS

Sampling

Samples were taken from edible parts of *Detarium microcarpum*, *Gardenia erubescens*, *Parkia biglobosa*, *Adansonia digitata*, *Lannea microcarpa* and *Vitellaria paradoxa* fruit pulp, *Boscia senegalensis* seeds and from *A. digitata* leaves. Fruits, seeds and leaves were collected from different localities in Burkina Faso including Noumoudara (10 ° 58'60" N, 4 ° 24'W), Sakoinsé (12 ° 11'N, 1 ° 57'W), Pobé-Mengao (14 ° 3'N, 1 ° 32'W), Kokologho (12 ° 11'N, 1 ° 53'W), Saria (12 ° 16'N, 2 ° 9'W), Tanghin-Dassouri (12 ° 16'10'"N, 1 ° 42'55" W) and Boulbi (12 ° 14'16" N, 1 ° 31'33'W). Fruits which are directly consumed without any transformation were directly collected in trees. The products which need transformation and conservation were collected in household in order to identify their effective nutritional value. The fruits of *L. microcarpa*, *G. erubescens*, *V. paradoxa* were collected directly from trees in their predilection area. For each product (fruits, pulp, seed and leaves), three samples from three different trees were collected. The fruits of *D. microcarpum* and *A. digitata* and the pulp of *P. biglobosa* were collected in three households for each. Edible parts of samples were separated and ground using a porcelain mortar and packed in opaque plastic bags in laboratory. Packed samples were kept in cool temperature until required successively for analysis.

Proximate composition

Proximate analysis of samples was conducted using the conventional procedures described by the Association of Official Analytical Chemists (AOAC, 2005): Dry matter by drying at 105°C overnight, ash by incineration at 550°C for 12 h, crude protein (N × 6.25) by the Kjeldahl method and crude fat content by Soxhlet extraction using n-hexane. Metabolizable energy values (kcal/100 g) were calculated by multiplying the grams of protein, fat, and carbohydrate by the factors of 4, 9, and 4 kcal/ g, respectively. The pH was established using HANNA pH-meter (Hanna HI 991300) at 25°C. Titrable acidity was determined by titrating 2.0 g of samples in 100 ml of water with 0.1 M NaOH using phenolphthalein as the indicator and was calculated as percentage citric acid. The total sugars were measured according to the sulfuric orcinol method as described by Montreuil and Spik (1969).

Mineral content

Mineral elements of the samples were determined according to procedures described by Walinga et al. (1989). The sample (1 g) was digested with 4 ml of a mixture (ratio 7:1) of perchloric acid ($HClO_4$, 60%) concentrated sulfuric acid and 15 ml of concentrated nitric acid. After complete digestion, the product was cooled, filtered and the volume adjusted to 50 ml. For determination of calcium, 0.2 ml of the filtered solution was diluted with 4.8 ml of lanthane (La_2O_3, 1%). For the other minerals, the dilutions were made with distilled water. Potassium was measured using a flame photometer (Corning 400, Essex, England); phosphorus was determined with a Skalar auto analyzer (Skalar, Breda, The Netherlands) and all other minerals with an atomic absorption spectrophotometer (Perkin Elmer Analyst 100).

Amino acids

The profile and the amount of total amino acids were determined by reverse phase HPLC, using the Pico-Tag system described by Bidlingmeyer et al. (1984). Samples were first defatted and hydrolyzed. For hydrolysis, 0.4 g of defatted sample placed in a flask and 15 ml of 6 M HCl added and the whole placed in an oven at 110°C for overnight. The sample are subsequently cooled to room temperature and transferred to a 50 ml volumetric flask and filled with Milli-Q water. Approximately, 1 ml of the diluted solution is homogenized and filtered through a filter of 0.45 µm. An aliquot of 10 µl of the solution is placed in reaction tube and dried for 15 min with the Picotag Workstation. The sample was then re-dissolved in 10 µl of re-drying solution (ethanol: water: triethylamine, 2:2:1 volume). They were dried again for 15 min and finally derivatized with 20 µl phenylisothiocyanate reagent (ethanol: water: triethylamine: phenylisothiocyanate, 7:1:1:1) for 20 min at room temperature. Excess reagent was removed with the aid of vacuum for 45 min in Pico-Tag Workstation. Derivatized samples were dissolved in 100 µl PicoTag Sample diluent solution (WAT088119). Analysis (identification and quantification) of amino acids performed using a Waters C18 column under the conditions described by Bidlingmeyer et al. (1984).

Briefly, 40 µl of aliquot was injected onto the column. Quantitation of amino acids was performed using a Waters C18 column (3.9 × 150 mm) with gradient conditions as described elsewhere. Derivatized amino acids were eluted from the column with increasing concentrations of acetonitrile. The eluate was monitored at 254 nm and the areas under the peaks were used to calculate the concen-

Table 1. Proximate composition (g/100 g DM) and energy value (Kcal/100 g DM) of dried products of tree species.

Trees specie	Analyses					
	Dry Matter	Ash	Protein	Lipid	Carbohydrate	Energy
Detarium microcarpum (pulp)	95.07±0.1[a]	3.26±0.01[d]	4.65±0.10[c]	1.70±0.01[c]	74.65±0.01[b]	335.5
Boscia senegalensis (seeds)	92.54±0.0[c]	1.16±0.10[f]	24.23±0.1[a]	1.11±0.08[d]	45.29±1.39[e]	288.07
Adansonia digitata (pulp)	91.31±0.14[d]	5.27±0.01[b]	2.00±0.01[e]	1.55±0.40[bdc]	72.30±0.52[c]	311.15
Adansonia digitata (leaves)	93.02±0.8[b]	11.76±0.1[a]	14.80±0.6[b]	4.65±0.12[a]	57.04±1.59[d]	329.21
Gardenia erubescens (pulp)	23.7±0.80[f]	2.54±0.08[e]	1.10±0.20[f]	1.19±0.01[d]	6.70±0.11[f]	41.9
Parkia biglobosa (pulp)	90.06±0.1[e]	4.98±0.01[c]	3.78±0.01[d]	1.99±0.05[b]	79.73±1.69[a]	351.95

Values are Mean ± Standard Deviation for at least 3 samples in triplicate. Values with different letters in the same column are significantly different at P < 0.05.

Table 2. Physical parameters of aqueous fruit pulp.

Parameter	*Lannea microcarpa*	*Vitellaria paradoxa*	*Boscia senegalensis*
Acidity (% citric acid equivalent)	0.45± 0.03[a]	0.17± 0.01[b]	1.29±0.03[c]
pH	4.60 ± 0.05[a]	5.85 ± 0.01[b]	2.78±0.02[c]
Degree Brix (°Brix)	23.8±3.7[a]	30.5±1.7[b]	22.58±0.15[a]

Values are Mean ± Standard Deviation for at least 6 samples in duplicate. Values with different letters in the same row are significantly different at P < 0.05.

Table 3. Minerals content of samples.

Trees specie	Ca	Mg	K	P	Mn	Fe	Zn	Cu
	Kg DM				Mg/Kg DM			
Parkia biglobosa (pulp)	1.61[a]	1.57[a]	19.24[a]	1.02[a]	96.99[a]	88.83[a]	17.40[a]	3.78[a]
Adansonia digitata (pulp)	2.49[b]	1.48[a]	22.01[b]	0.45[b]	11.79[b]	65.53[b]	19.36[b]	5.16[b]
Adansonia digitata (leaves)	14.34[c]	4.14[b]	16.75[c]	0.37[b]	95.44[a]	401.98[c]	12.22[c]	5.36[c]
Boscia senegalensis (seeds)	0.44[d]	0.08[c]	4.45[d]	1.20[a]	9.59[c]	826.97[d]	25.98[d]	4.32[a]
Detarium microcarpum (pulp)	1.06[e]	0.98[d]	28.80[e]	0.63[c]	39.65[d]	100.72[e]	16.79[a]	3.29[a]
Lannea microcarpa (pulp)	3.56[f]	2.38[e]	11.73[f]	0.30[b]	9.07[c]	91.61[a]	10.57[e]	5.13[b]
Vitellaria paradoxa (pulp)	2.33[b]	0.94[d]	11.73[f]	0.16[d]	3.68[d]	88.10[a]	5.75[f]	2.93[a]

Values are Means for at least 3 samples in triplicate. Values with different letters in the same row are significantly different at P < 0.05.

trations of the unknowns using a Pierce Standard H amino acid calibration mixture (Rockford, IL).

Data analysis

Data were computed in Excel and analyzed with XLSTAT version 7.5.2. Statistical analysis was focused on principal component analysis (PCA) and analysis of variance (ANOVA). These analysis were performed with the software "XLSTAT Version 7.5.2" with a risk of error p = 5%.

RESULTS

The proximate composition and Energy value, Physical parameters of aqueous fruit pulp, mineral content and amino acid profile of the sample are shown in Table 1, 2, 3 and 4, respectively. The total carbohydrates content of products ranged from 6.7±0.11 to 79.73±1.69 (Table 1). The lowest value of total carbohydrates was found in *G. erubescens* fruit pulp while the pulp of *P. biglobosa* had shown the highest content. The pulp of *P. biglobosa* (79.73±1.69), *A. digitata* (72.3±0.52) and *D. microcarpum* (74.65±0.01) had the highest content compared to other samples. The leaves of *A. digitata* shown the highest content of lipids (4.65±0.12), while the seeds of *B. senegalensis* shown the lowest content (1.11±0.08). The results of the present study (Table 3) revealed that the studied products are good source of minerals. The amino acids content of *A. digitata* leaves and *B. senegalensis*

Table 4. Amino acids profiles of *Adansonia digitata* leaves and *Boscia senegalensis* seeds (mg/g of proteins).

Amino acid (Mg/g protein)	Seeds of *B. senegalensis*	Leaves of *A. digitata*
Asp	31.03 ± 7.79	24.90 ± 3.61
Glu	33.67 ± 8.36	24.90 ± 3.61
Ser	15.90 ± 3.47	10.27 ± 1.80
Gly	12.47 ± 3.78	11.67 ± 1.14
His	5.27 ± 1.15	5.80 ± 1.97
Arg	43.50 ± 12.24	14.67 ± 1.89
Thr	7.97 ± 1.76	10.27 ± 1.80
Ala	11.37 ± 2.06	14.67 ± 1.89
Pro	42.03 ± 12.62	31.13 ± 4.44
Tyr	7.97 ± 1.76	8.93 ± 1.29
Val	14.37 ± 4.88	11.63 ± 1.10
Met	2.63 ± 0.58	4.47 ± 0.64
Cys	2.63 ± 0.58	-
Ile	7.97 ± 1.76	8.93 ± 1.29
Leu	21.23 ± 5.25	14.67 ± 1.89
Phe	15.17 ± 4.37	11.63 ± 1.10
Lys	7.97 ± 1.76	11.63 ± 1.10

seeds are reported in the Table 4.

DISCUSSION

Proximate composition and energy value of the food tree species

In general, variability in the chemical content are reported to be dependent on soil (habitat), climatic variations, genetic factors, maturity and the storage conditions of the samples as reported elsewhere by Chadare et al. (2009); Diop et al. (2005); Osman (2004). The ash content of the pulp of *A. digitata* in a current study is similar with the 4.97±0.02 reported by Compaoré et al. (2011) in an earlier study in Burkina Faso. For the pulp of *P. biglobosa*, the value found was similar to the value reported by Compaoré et al. (2011). The ash content of the fruits of *D. microcarpum* is fairly similar to 3.04 to 3.1 reported by Parkouda et al. (2007). For *G. erubescens,* the ash content (2.54±0.08) found was lower than that of the value (3.69-4.56) reported by Parkouda et al. (2007). The leaves of *A. digitata* had ash content (11.76±0.1) between the values of 8 and 16 g/100 g reported by Diop et al. (2005).

Statistically, the protein content of the products analyzed was significantly different. Among the studied products, *A. digitata* leaves and *B. senegalensis* seeds had good level of protein (Table 1) and *B. senagalensis* seeds can be considered as potential source of protein. The leaves of *A. digitata* protein content is similar to that value (10.3 to 15 g/100 g) reported by Diop et al. (2005) but higher than the value (10.3 g/100 g) found in an earlier study in Burkina Faso by Glew et al. (1997). The pulp of *A. digitata* has protein content lower than that of the value (5.23±0.03) reported by Compaoré et al. (2011). The protein content of *P. biglobosa* pulp was similar to the result reported by Parkouda et al. (2007) and lower than the value (5.37±0.07) reported by Compaoré et al. (2011). The *B. senegalensis* seeds proteins content is higher than the value (20.62) reported by Parkouda et al. (2007). The protein content of *D. microcarpum* fruits pulp are similar to the value reported by Kouyaté et al. (2009) but higher than the value (2.86) found earlier in Burkina Faso by Parkouda et al. (2007). As shown in Table 1, the lipids content ranged between 1.11±0.08 to 4.65±0.12 with the *B. senegalensis* seeds having the lowest value while the leaves of *A. digitata* had the highest content. Apart from *A. digitata* leaves, all the samples had fairly similar content of lipids. The *P. biglobosa* and *A. digitata* pulps lipids content are similar to the results reported earlier by Compaoré et al. (2011). *D. microcarpum* and *G. erubescens* pulp had lipid levels about ten times higher than previous study (Parkouda et al., 2007) who reported value of 0.1%. Lipid content of *B. senegalensis* seeds is similar to the previous reported by Parkouda et al. (2007) but lower than the value (3.7±0.8 g/100 g) reported in *B. senegalensis* seeds study in Niger by Kim et al. (1997). The leaves of *A. digitata* have a fat content between the values (2.3 to 10 g/100 g) reported by Diop et al. (2005).

The pulp of *P. biglobosa* and *A. digitata* from this study had higher levels than those reported by Compaoré et al. (2011) who found respectively 67.66±0.05 and 67.8±2.1. The pulp of *D. microcarpum* and *G. erubescens* had total carbohydrates content lower than those reported by Parkouda et al. (2007) while the *B. senegalensis* seeds had total carbohydrates content similar to that found by Parkouda et al. (2007). The total carbohydrates content of the *A. digitata* leave found (57.04±1.59) between the values (13.8 to 70) reported by Diop et al. (2005). The value of calculated metabolizable energy was comprised between 41.9 to 351.9 kcal/100 g. The metabolizable energy found in *A. digitata* pulp is lower than the value (320.3±4.4 kcal/100 g) reported by Osman (2004)

Physical parameters of aqueous fruit pulp

The most acidic was *S. senegalensis* and this may be responsible for the sour taste of this pulpy fruit. The low level of acidity in the *V. paradoxa* pulp can be explained by the fact that they do not contain enough free organic acids. Organic acids play an important role in the sensorial quality of product because the flavor is essentially a balance between sugar content and acidity (Neta et al., 2007). Indeed sugars and organic acids are two parameters used as indicators of maturity or ripeness of the fruit

(Mahmood et al., 2012).

Mineral content of food tree species

The wide differences found in the chemical content of the current study can be attributed to soil (habitat), climatic variations, genetic factors, maturity and the storage conditions of the samples as reported by Chadare et al. (2009); Diop et al. (2005); Osman (2004). The pulp of *A. digitata* and *P. biglobosa* had higher levels of manganese compared to the report of Compaoré et al. (2011) who reported 0.6 and 78.5 mg/kg, respectively. The manganese content of the leaves of *A. digitata* is higher than the value 31 to 89 mg/kg and 31 mg/kg, respectively, reported by Diop et al. (2005); Glew et al. (1997). The iron content of the *A. digitata* pulp and *P. biglobosa* pulp are lower than those (149 mg/kg and 1030 mg/kg) respectively reported by Compaoré et al. (2011). The Iron content of the leaves of *A. digitata* was between the values (150 to 490 mg/kg) reported by Diop et al. (2005). The iron content of *V. paradoxa* pulp and *D. microcarpum* pulp are higher than those reported by Parkouda et al. (2007) respectively 45.8 and 61.5 mg/kg. The iron content of seeds of *B. senegalensis* was much higher that the values of 31.2 and 44.1 to 61.1 mg/kg reported respectively by Parkouda et al. (2007); Kim et al. (1997). This difference may be due to the processing methods applied by processors before storage; indeed collected seeds are generally extracted exhaustively with water to remove bitter components and possible toxic substances (Kim et al., 1997).

The zinc content of the leaves of *A. digitata* is lower than that reported by Glew et al. (1997); Diop et al. (2005) who found respectively 18.7 and 19 mg/kg. The pulp of *P. biglobosa* had a zinc content lower than that of (30.1 mg/kg) reported by Compaoré et al. (2011) while the zinc content of *A. digitata* was higher than that of the value (15.4 mg/kg) reported by Compaoré et al. (2011) and 18 mg/kg reported by Osman (2004). The zinc content of seeds of *B. senegalensis* and pulp of *V. paradoxa* are lower than those reported by Parkouda et al. (2007) respectively 33.5 and 22.3 mg/kg. The pulp of *A. digitata* and *P. biglobosa* had copper contents much lower than those values (67.3 and 252 mg/kg) reported by Compaoré et al. (2011). Osman (2004) reported value of 18 mg/kg for the *A. digitata* pulp. The copper content of the leaves of *A. digitata* was between the value (1 to 12 mg/kg) reported by Diop et al. (2005). The calcium content of *P. biglobosa* pulp are similar to the value (1.1 g/kg) reported by Compaoré et al. (2011). The calcium content (2.49 g/kg) of the pulp of *A. digitata* is similar to the value (2.9 g/kg) reported by Osman (2004) but lower than the values (3.1 g/kg) reported by Compaoré et al. (2011). For the leaves of *A. digitata* the value found (14.34 g/kg) is comprised between the values (3.1 to 40.2 g/kg) reported by Diop et al. (2005). The calcium content of *B. senegalensis* seeds was similar to the values (0.17 to 0.5 g/kg) found by Kim et al. (1997).

The *A. digitata* pulp had value (1.48 g/kg) similar to the value (1.55 g/kg) reported by Compaoré et al. (2011) but lower than the value (2.1 g/kg) reported by Glew et al. (1997) and higher than the value (0.9 g/kg) reported by Osman (2004). For the leaves of *A. digitata* the content is between the values (3.1 to 5.5 g/kg) reported by Diop et al. (2005). The magnesium content of seeds of *B. senegalensis* (0.08 g/kg) is lower than the value (0.6 g/kg) reported by Parkouda et al. (2007). The seeds of *B. senegalensis* showed a value 4.45 g/kg which is lower than the value (5.0 to 8.9 g/kg) found by Kim et al. (1997). The potassium content of the leaves of *A. digitata* is similar to that reported by Diop et al. (2005). The pulp of *A. digitata* and *P. biglobosa* had potassium contents higher than values reported by Compaoré et al. (2011). Earlier study in Saudi Arabia, Osman (2004) reported potassium value of 12.4 g/kg for *A. digitata* pulp. The pulp of *A. digitata* and *P. biglobosa* have phosphorus levels lower than those reported by Compaoré et al. (2011). The leaves of *A. digitata* phosphorus content was between the values (0.3 to 6.7 g/kg) reported by Diop et al. (2005).

As reported by Elinge et al. (2012), minerals contribute to diverse functions of human body such as blood pressure, fluid balance and blood volume regulation (sodium); nutrients passage through cell walls and muscle contraction (calcium); releasing of parathyroid hormone and tissue respiration (magnesium); buffering of the human body fluid for metabolism and facilitation of the nutrient crossing other cell membrane (phosphorus); formation of blood and transfer of oxygen and carbon dioxide from one tissue to another (iron); important role in all mental functions and transfer of oxygen from lungs to cells (manganese); boosting the health of hair and playing sensorial such as ability to tastes, sense and smell (zinc). Based on the results obtained, these products consumed during the shortage periods have potential to contribute to alleviate the malnutrition during this period if they are available in quantity and quality.

Amino acids of *A. digitata* leaves and *B. senegalensis* seeds

The results showed that the leaves of *A. digitata* and seeds of *B. senegalensis* had a good profile of amino acids. However, cysteine is the limiting amino acid in the leaves of *A. digitata*. The values of the amino acids obtained from the leaves of *A. digitata* in a current study are higher than those reported by Glew et *al.* (1997).

Conclusion

The present study distinguished some wild tree species which can be used as source of protein (seeds of *B.*

senegalensis), as a source of metabolizable energy (*D. microcarpum*, pulp of *P. biglobosa* and *A. digitata*) or minerals. Nutritionally, these products could contribute positively to the minerals intake. Most of the minerals determined are essential elements for normal body functioning. During food shortage period, consumption of these products will help to overcome nutrients deficiency in urban and rural areas.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Tissue diversity in respiratory metabolism and free radical processes in embryonic axes of the white mangrove (*Avicennia marina* L.) during drying and wet storage

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Species diversity in responses to desiccation in plants is well studied and documented. However, organ and tissue variability in plant dehydration responses is not as well investigated and understood. Therefore, the responses of whole axes, hypocotyls, root primordia and plumules of white mangrove to drying and wet storage were monitored. Increasing the rate of drying lowered the critical and lethal water concentrations for survival as assessed by germination capacity and tetrazolium staining. Dehydration and hydrated storage were generally associated with decrease in activities of phosphofructokinase (PFK) and malate dehydrogenase and levels of nicotinamide adenine dinucleotide and an increase in the levels of hydroperoxides in whole axes, hypocotyls, root primordia and plumules and membrane damage in axes. Increase in the activities of superoxide dismutase and catalase and decrease in the activities of glutathione reductase and amounts of ascorbate accompanied drying and moist storage in all tissues, in general. Apart from the activity of PFK, the plumules showed the highest activities and quantities of all the enzymes and compounds among the tissues during desiccation and wet storage. It is possible that this tissue, despite its relatively small size and volume plays disproportionately an important role in the events described. Nonetheless, it is likely that physical rather than metabolic damage underlined loss of viability as it occurred at high water concentration.

Key words: Antioxidant, *Avicennia marina* desiccation, drying rate, free radical processes, lipid peroxidation, metabolism, respiration seed survival.

INTRODUCTION

Avicennia marina (Forssk.) is an Indo-Pacific tropical intertidal estuarine (mangrove) tree that produces desiccation-sensitive (recalcitrant) seeds. These seeds are shed around the autumn equinox and can be carried to the inland fringes of the mangrove by the equinoctial high spring tide. Subsequent inundation may not occur for several months and so these seeds will die from desiccation damage. Knowledge of the response of *A. marina* seeds to desiccation contributes to an understanding of the biology of a keystone mangrove species. Furthermore, many tropical and subtropical species produce recalcitrant seeds, which have implications

in biodiversity conservation. Desiccation tolerant seeds are generally stored in the dry state and the desiccation sensitivity of recalcitrant seeds precludes their storage by conventional means. This impact negatively on attempts at long-term storage of germplasm represented by these seeds, which, in turn, has implications for long-term biodiversity conservation. It is possible to store recalcitrant seeds at their shedding water content (hydrated storage), but this is strictly a short-term measure as viability is rapidly lost (Motete et al., 1997). The studies on the response of *A. marina* to drying reported here contribute to an increased knowledge of the species, of seed recalcitrance, and the ability to conserve the germplasm of species producing these seeds

In addition to physical stresses that desiccation-sensitive seed tissues incur as a consequence of dehydration, they are subjected to metabolic damage. It has been argued that desiccation differentially affects the activities of various enzymes (Farrant et al., 1985; Leprince et al., 1993a; 1994; 1995; 1999; 2000; Côme and Corbineau, 1996; Leprince and Hoekstra, 1998; Ntuli et al., 2011) and hence results in metabolic imbalance (Finch-Savage et al., 1993; reviewed by Vertucci and Farrant, 1995; Leprince et al., 2000; Ntuli et al., 2011). Of a particular interest in this regard, is respiratory metabolism. For instance, it has been shown that phosphofructokinase (PFK) and malate dehydrogenase (MDH) were slightly and mildly affected by dehydration, respectively, while the glucose-6-phosphate dehydrogenase (G6PDH) and NADH dehydrogenase of NADH-ubiquinone (coenzyme Q) reductase (complex I) were extremely sensitive to desiccation in germinating maize (Leprince et al., 1992; 1993a; 1994). In contrast, Carpenter et al. (1987) observed that PFK was highly sensitive to *in vitro* desiccation. In this regard, it is noteworthy that the cause of the differences in sensitivity among enzymes is unknown. More recently, Song et al. (2009) reported a decline in the activity of malate dehydrogenase in recalcitrant *Antiaris toxicaria* axes and orthodox *Zea mays* embryos during dehydration.

It has been suggested that desiccation-induced disruption of the electron transport chain of mitochondrial and microsomal membranes between the ubiquinone pool and cytochrome oxidase may result in more leakage of electrons from the electron transport chain than normal, thus increasing the generation of free radical species, which are associated with lipid peroxidation (Leprince et al., 1990; 1994; 1995; Hendry et al., 1992; Hendry, 1993). More recently, Song et al. (2009) reported a decline in the activity of cytochrome c oxidase in recalcitrant *Antiaris toxicaria* axes and orthodox *Zea mays* embryos during dehydration. Roach et al. (2010) have demonstrated extracellular superoxide production in response to desiccation in recalcitrant *Castanea sativa* seeds. However, it remains unclear whether oxidative damage is a cause or consequence of tissue damage and/or death (Hendry et al., 1992; reviewed by Leprince and Golovina, 2002).

One of the mechanisms contributing to desiccation tolerance is the nature and efficiency of free radical processing systems (reviewed by Crowe et al., 1992; Leprince et al., 1993b; Horbowicz and Obendorf, 1994; Vertucci and Farrant, 1995; Close, 1996; Berjak and Pammenter, 2008; Pammenter and Berjak, 1999; Buitink et al., 2002). For instance, Hendry et al. (1992) demonstrated that dehydration of desiccation-sensitive embryonic axes of *Quercus robur* resulted in decreases in the activities of superoxide dismutase (SOD), glutathione reductase (GR), ascorbate peroxidase (APO) and the levels of α-tocopherol. In contrast, there was an increase in the levels of ascorbate (AsA) during dehydration. However, guaicol peroxidase (GPO) was not affected by drying. No catalase (CAT) activity was detected. More recently, Song and co-workers have shown a decline in activities of SOD, APO, CAT, GR, and dehydroascorbate reductase (DHAR) and SOD, APO and CAT in axes of Chineese wampee during dehydration (Huang et al., 2009, respectively).

Several studies have shown that rapid drying of desiccation-sensitive seeds permits survival to lower water contents than slow drying (Berjak et al., 1984; Farrant et al., 1985; Pritchard, 1991; Walters et al., 2001; Wesley-Smith et al., 2001; Huang et al., 2009; Ntuli et al., 2011). It has been proposed that such behaviour is consequent upon rapid drying removing water sufficiently fast to reduce the accumulation of damage resulting from aqueous-based deleterious reactions (Pritchard, 1991). To achieve the rates of drying to demonstrate this effect requires small pieces of tissue, and so the embryo or embryonic axis must be excised from the seed.

Although the responses of plant tissue to dehydration have been studied in a number of species, the responses of the individual seed components have been less frequently reported. This is important when studying recalcitrant seeds as different components of the seed or embryonic axes may dry at different rates and/or show differential sensitivities to dehydration.

A. marina is one of the most desiccation-sensitive

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Abbreviations: A/GPO, Ascorbate/guaicol peroxidase; **AsA,** ascorbic acid; **CAT,** catalase; **GR,** glutathione reductase; **MDH,** malate dehydrogenase; **NAD(H),** (reduced) nicotinamide adenine dinucleotide; **PFK,** phosphofructokinase; **SOD,** superoxide dismutase; **TZ,** tetrazolium.

recalcitrtant species recorded (Berjak et al., 1984) and so its response to dehydration is of particular importance. This report examines the response of respiratory metabolism and free-radical processing of whole axes, hypocotyls, root primordia and plumules of seeds of *Avicennia marina* subjected to rapid or slow drying or hydrated storage.

MATERIALS AND METHODS

Plant material

Newly-shed propagules of white mangrove (*Avicennia marina* L.) were collected from various trees in Isipingo Beach and Beachwood Nature Reserve in Durban, South Africa. Embryonic axes were excised from cotyledons and accumulated on moist filter paper in closed Petri dishes.

Treatments

Embryonic axes were partitioned into three samples. Each sample was aseptically placed in sterile containers over:

1) Activated silica gel with a fan mounted (rapid drying [close to 0% RH]),
2) A saturated solution of sodium chloride (slow dehydration [75 ± 1% RH]) or distilled water (wet storage [100% RH]). The containers were stored at 15 ±1°C.

Water concentration determinations

Water contents were determined gravimetrically after drying axes in the oven at 80°C for 48 h. Masses were determined periodically during dehydration and wet storage. The water contents are reported as means ± SE for five individual axes, expressed on a dry mass basis (g g^{-1} [dm]).

Viability tests

To minimize the effects of imbibitional damage, dehydrated embryonic axes were moistened on damp filter paper in Petri dishes overnight at 20°C before being subjected to the germination, tetrazolium and conductivity tests. To assess germination capacity, axes were cultured in Petri dishes on half MS medium (Musharige and Skoog, 1962) supplemented with 0.3 g l^{-1} sucrose, photoperiod 16 h, under sterile conditions for a period of 20 days at room temperature. Axes were scored as germinated when they showed greening and/or elongation and/or expansion.

Apparent embryonic axis viability was determined by the tetrazolium test. Twenty moistened radicles were cut through longitudinally, soaked in 1% (w/v) 2, 3, 5- triphenyltetrazolium chloride solution for 24 h in the dark at 20°C, and scored using intensity and location of staining as criteria (International Seed Testing Association, 1996).

Electrolyte leakage from five replicates of individual moistened embryonic axes was measured using a multi-cell conductivity meter (CM100; Reid and Associates cc, Durban) over 12 h. All measurements were made at 2 V whilst axes were immersed in 3 ml of distilled water. Leakage was recorded as the highest reading over the measurement period. The results are reported as means ±SE of the five replicate axes.

Respiratory enzymes assays

Phosphofructokinase (PFK) activity was determined according to Leprince et al. (1993a). Root primordia, hypocotyls, plumules and whole axes (*c*. 5 mg dry matter) were homogenised to a fine powder under liquid nitrogen using a pestle and mortar. Soluble proteins were extracted from the frozen powder in 5 ml of 50 mM Tris-HCl (pH 7.6) in the presence of 0.1% polyvinylpyrolidone (PVP) (Hofmann and Kopperschläger, 1982) and the homogenate centrifuged at 8 000 *g* for 5 min. An aliquot of 2 ml of the supernatant was then transferred to 1 ml of a mixture of 0.2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM fructose-6-phosphate, 5 mM MgCl2, 0.6 mM ATP, 0.33 U ml^{-1} aldolase, 10 U ml^{-1} triose phosphate isomerase, 1 U ml^{-1} glycerophosphate de-hydrogenase and 0.2 mM NADH in Tris (pH 7.6) buffer. Activity was monitored by measuring the formation of fructose-1, 6-bisphosphate, as indicated by a coupled NADH-dependent reduction of dihydroxyacetone phosphate to glycerol-3-phosphate, as the change in absorbance at 340 nm over 3 min. Malate dehydrogenase (MDH) activity was monitored using the procedure of Leprince et al. (1993a). Radicles (*c*. 5 mg dry mass) were homogenised to a fine powder under liquid nitrogen using a mortar and pestle. Soluble proteins were extracted from frozen powder in 5 ml of 50 mM potassium phosphate buffer (pH 7.4) in the presence of 0.1% PVP (Siegel and Bing, 1956). The homogenate was then centrifuged at 8 000 *g* for 5 min. An aliquot of 0.1 ml of the supernatant was added to 2 ml of 0.1 M phosphate buffer (pH 7.55) and 0.1 ml of 2 mg/ml NADH. After 10 min, 0.1 ml of 0.5 M oxaloacetate in 0.1 M phosphate buffer (pH 7.0) was added. Activity was determined by observing the change in optical density of NADH at 340 nm over a 3 min period.

Oxidised nicotinamide adenine dinucleotide (NAD) assay

Root primordia, hypocotyls, plumules and whole axes (*c*. 5 mg dry mass) were homogenized in 5ml of 0.2 M HCl following homogenization to a fine powder under liquid nitrogen in the mortar with a pestle, heated in a boiling water bath for 5 min, cooled in an ice bath and centrifuged at 14 000 *g* for 10 min (Zhao et al., 1987). An aliquot of 0.5 ml of the supernatant was then transferred to 1.0 M Bicine-NaOH buffer (pH 8.0) and neutralized with 0.2 M NaOH in the dark (Matsumura and Miyachi, 1980). Following the addition of 0.1 ml each of 40 mM EDTA, 4.2 mM 3-(4,5 dimethyl-thiazoyl-2)-2,5-diphenyltetrazolium bromide (MTT), 16.6 mM phenol ethosulfate and 5.0 M ethanol, 0.1 ml of 500 U ml^{-1} alcohol dehydrogenase was added after 5 min at 37°C. The level of NAD was determined by measuring the rate of reduction of MTT as absorbance at 570 nm after 30 min.

Lipid peroxidation assessment

Lipids were extracted from radicals (*c*. 5 mg dry mass) that had been homogenized under liquid nitrogen in a mortar with a pestle, in 5 ml of dichloromethane/methanol (2:1 v/v) containing butylated hydroxytoluene (50 mg l^{-1}) according to Hailstones and Smith (1988). Following centrifugation at 1500 *g* for 5 min, 1 ml of 0.014 M ferrous chloride (FeCl2) was added to 2 ml of the lipid extract in dichloromethane/methanol and shaken. Twenty µL of 30% potassium thiocyanate (KSCN) were then added. Hydroperoxide levels were estimated by the oxidation of Fe^{2+} as the absorbance recorded at 505 nm.

Antioxidant assays

Superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) were extracted in 10 ml of 50 mM potassium

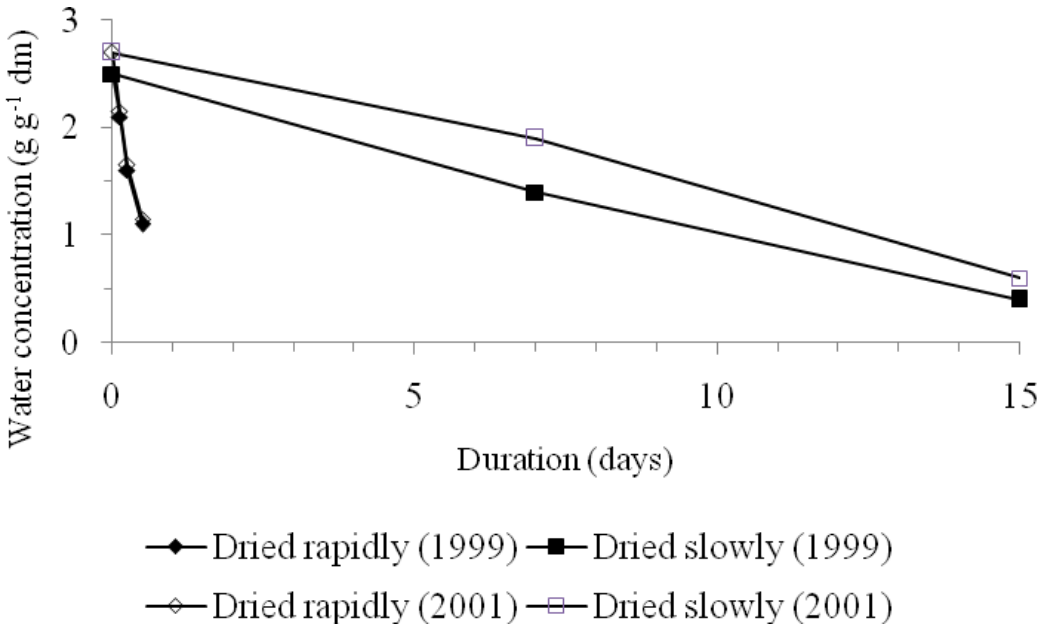

Figure 1. Water concentrations of embryonic axes of *Avicennia marina* during desiccation. (Error bars represent means ± SE, n = 5, all smaller than symbols).

phosphate (pH 7.0), 0.25% (w/v) Triton X-100 and 1% (w/v) polyvinylpolypyrolidone, following homogenisation of Root primordia, hypocotyls, plumules and whole axes (*c*. 5 mg dm) to a fine powder under liquid nitrogen in the mortar with a pestle, following the procedures of Mishra et al. (1993; 1995). The homogenate was then centrifuged at 8 000 g for 15 min. For SOD, the reaction was performed with an aliquot of 2 ml of the supernatant and 1 ml of 50 mM potassium phosphate (pH 7.8), 0.1 mM EDTA containing 18 µM cytochrome *c* and 0.1 mM xanthine and the reaction started with addition of 0.02 ml of 1 U ml-1 of xanthine oxidase (McCord and Fridovich, 1969; Schoner and Krause, 1990). Activity was monitored by measuring the rate of reduction of cytochrome c as the change in absorbance at 550 nm.

CAT was assayed in an aliquot of 2 ml of the supernatant added to 1 ml of 50 mM potassium phosphate containing 11 mM H_2O_2 (pH 7.0). Activity was determined as the decomposition of H2O2 by the decrease in absorbance at 240 nm (Aebi, 1983). GR was assayed in an aliquot of 2 ml of the supernatant added to 1 ml of 25 mM Tris- HCl containing 0.5 mM oxidised glutathione (GSSG) and 0.12 mM NADPH (pH 7.8) (Foyer and Halliwell, 1976). Activity was recorded by measuring the oxidation of NADPH as the decrease in absorbance at 340 nm.

AsA was extracted in 4 ml of 2.5 mM perchloric acid ($HClO_4$) following homogenisation of radicles (*c*. 5 mg dm) in liquid nitrogen in the mortar with a pestle according to Foyer *et al*. (1983). The homogenate was then centrifuged at 8 000 g for 5 min and neutralised to pH 5.6 with 1.25 M K_2CO_3. One ml of the extract was transferred to 1 ml of 0.1 M sodium phosphate buffer (pH 5.6) and reaction started with addition of 1 ml of 5 U ml^{-1} of ascorbate oxidase (Hewitt and Dickes, 1961). The level of AsA was estimated by measuring its oxidation as the decrease in absorbance at 265 nm.

For all the biochemical assays, the extractions were performed on five embryonic axes (*c*. 5 mg dm) in at least three replicates at 4°C. All the assays were carried out at 25°C. All chemicals were bought from Sigma and the units are as defined thereby. Results are reported as means □ □ SE of the replicate extractions and % change of the initial value.

Dry mass estimations

For the conductivity tests and all biochemical assays, dry mass was calculated from wet mass and water contents expressed on a dry mass basis. Wet mass was determined before the tests or assays were conducted.

Statistical analysis

Data were subjected to one-way ANOVA test. Where significant effects were found to occur, the Turkey multiple range test was subsequently used to distinguish among significantly deviating means.

RESULTS

Desiccation kinetics

Both slow and rapid drying showed almost linear dehydration. Whole axes reached a water concentration of 1.1 g g^{-1} dm in 12 h during rapid drying while a final water content of 0.29 g g^{-1} dm was attained in 15 days upon slow dehydration (Figure 1). As a result, rapid desiccation facilitated drying around 20 times more rapidly than slow dehydration, water contents remained constant during wet storage (data not shown).

Survival characteristics

Newly-shed axes registered 100% germination and tetrazolium staining for both harvests (Figures 2A and C). A 40% decrease in germination capacity occurred at *c*. 2.0 and 2.4 g g^{-1} dm during fast and slow drying of axes from

seeds harvested in 1999, respectively. A similar decline in germination capacity was observed at *c*. 2.0 and 2.6 g g^{-1} dm following rapid and slow dehydration of axes from the 2001 harvest, respectively. Total loss of the ability to germinate occurred at *c*. 0.3 and 0.7 g g^{-1} dm upon slow desiccation. Tetrazolium staining remained at 100% for both harvests during fast dehydration. Conversely, an abrupt decline occurred on slow drying at *c*. 1.4 and 2.0 g g^{-1} dm for 1999 and 2001 harvests, respectively. Germination capacity and tetrazolium staining decreased sharply after one week of wet storage for both harvests (Figures 2B and D). Loss of ability to germinate was complete after two weeks of hydrated storage.

Electrolyte leakage

Axes of *A. marina* recorded a mean electrolyte conductivity of *c*. 2.9 mS cm^{-1} g^{-1} dm after 12 h of leakage. Subsequently, there was gradual increase in electrolyte leakage with drying and wet storage (Figures 2E and F). The highest conductivity readings for axes dried rapidly and slowly were *c*. 4.5 and *c*. 15.9 mS cm^{-1} g^{-1} dm for the 1999 and 2001 harvests, respectively. Axes dried slowly recorded more leakage than those dried rapidly at a corresponding water content.

Respiratory metabolism

Phosphofructukinase (PFK)

A significant *c*. 70% decrease in the activity of PFK in whole axes accompanied the 40% decline in germination capacity during fast drying (Figures 3A and Table 1). Similarly, a significant *c*. 80% reduction in PFK activity in axes was associated with germination loss upon slow dehydration. There was no significant change in the activity of PFK in axes before the onset of germination loss during wet (Figure 3B).

The activity of PFK in hypocotyls was significantly diminished by *c*. 83% during germination loss upon rapid desiccation (Figure 3A). There were no significant changes in the PFK activity in hypocotyls during slow drying and hydrated storage. There were no significant changes in the activities of PFK in root primordial and plumules during fast and slow drying and moist storage.

Malate dehydrogenase (MDH)

A significant *c*. 40% decrease in the activity of MDH in axes occurred during loss of germination following slow drying (Figure 3C). Similarly a significant *c*. 85% decline preceded the onset of germination loss during wet storage (Figure 3D). MDH activity in hypocotyls, root primordia and plumules did not change significantly upon dehydration and hydrated storage.

Nicotinamide adenine dinucleotide (NAD)

No significant changes were observed in the levels of NAD in axes during fast drying and wet storage (Figures 3E and F). However, a significant *c*. 80% decrease in NAD level occurred following slow dehydration ($F = 427.40$ and $p = 0.04$).

There were no significant changes in the NAD level in hypocotyls upon desiccation and hydrated storage. A significant *c*. 90% decline in the level of NAD in root primordia accompanied germination loss during slow drying. The onset of germination loss was preceded by a *c*. 80% reduction in NAD level upon hydrated storage. No significant changes occurred in the level of NAD in plumules following fast dehydration and moist storage. Nonetheless, germination loss was associated with a *c*. 90% decrease in NAD level during slow desiccation.

Hydroperoxide levels

No significant changes in the levels of hydroperoxides in axes, root primordia and plumules were associated with drying and wet storage (Figures 3G and H). However, the hydroperoxide levels in hypocotyls increased significantly by *c*. 440% and 145% during fast and slow dehydration.

Antioxidant levels

Superoxide dismutase (SOD)

Significant *c*. 540 and 200% increases in the activity of SOD in axes occurred during fast drying and wet storage (Figures 4A and B). No significant changes in SOD activity in hypocotyls, root primordia and plumules were observed following dehydration and hydrated storage.

Catalase (CAT)

The activities of CAT did not significantly change in axes, hypocotyls and plumules during drying (Figures 4C and D). In contrast, a significant *c*. 360% in CAT activity in root primordia preceded the onset of germination loss following wet storage.

Glutathione reductase (GR)

The activity of GR in axes significantly decreased by *c*. 90% upon desiccation (Figure 4E). Similarly, the onset of the loss of germination was preceded by a *c*. 95% decline in GR activity during wet storage. Complete loss of germination was associated with a significant *c*. 40% reduction in the activity of GR in hypocotyls following slow dehydration. No significant changes in GR activity occurred in root primordia during drying and hydrated storage. A significant *c*. 90% decrease in the activity of GR in plumules accompanied loss of germination upon slow desiccation.

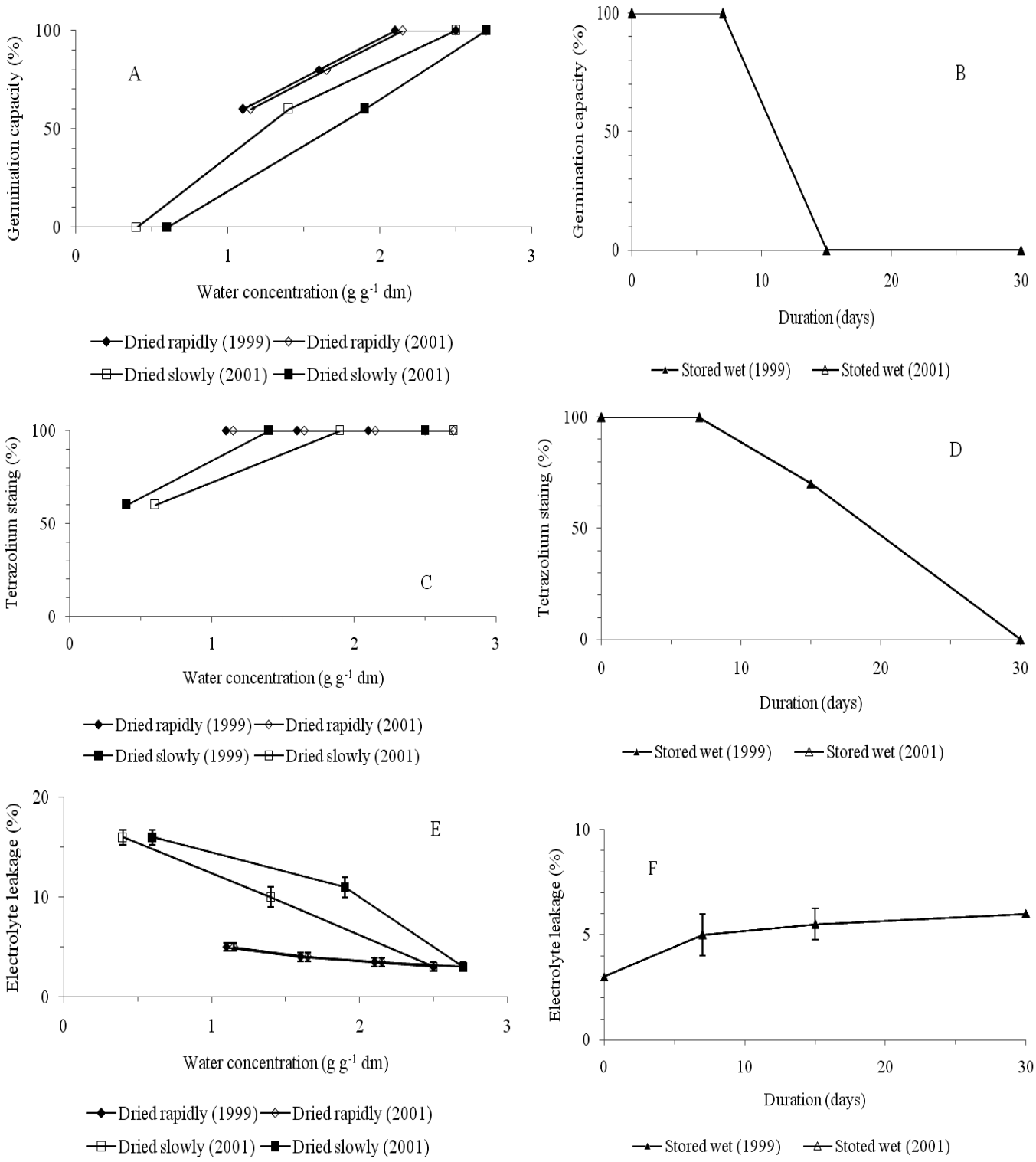

Figure 2. Germination capacity, tetrazolium staining and electrolyte leakage of *A. marina* axes following dehydration (A, C and E) or wet storage (B, D and F) in 1999 (open symbols) and 2001 (closed symbols). (Error bars represent means ± SE, n = 10, some smaller than symbols).

Ascorbic acid (AsA)

A significant *c*. 80% decrease in the levels of AsA in axes occurred during loss of germination during slow drying (Figure 4G). No significant changes were observed in the levels of AsA in hypocotyls, root primordia and plumules

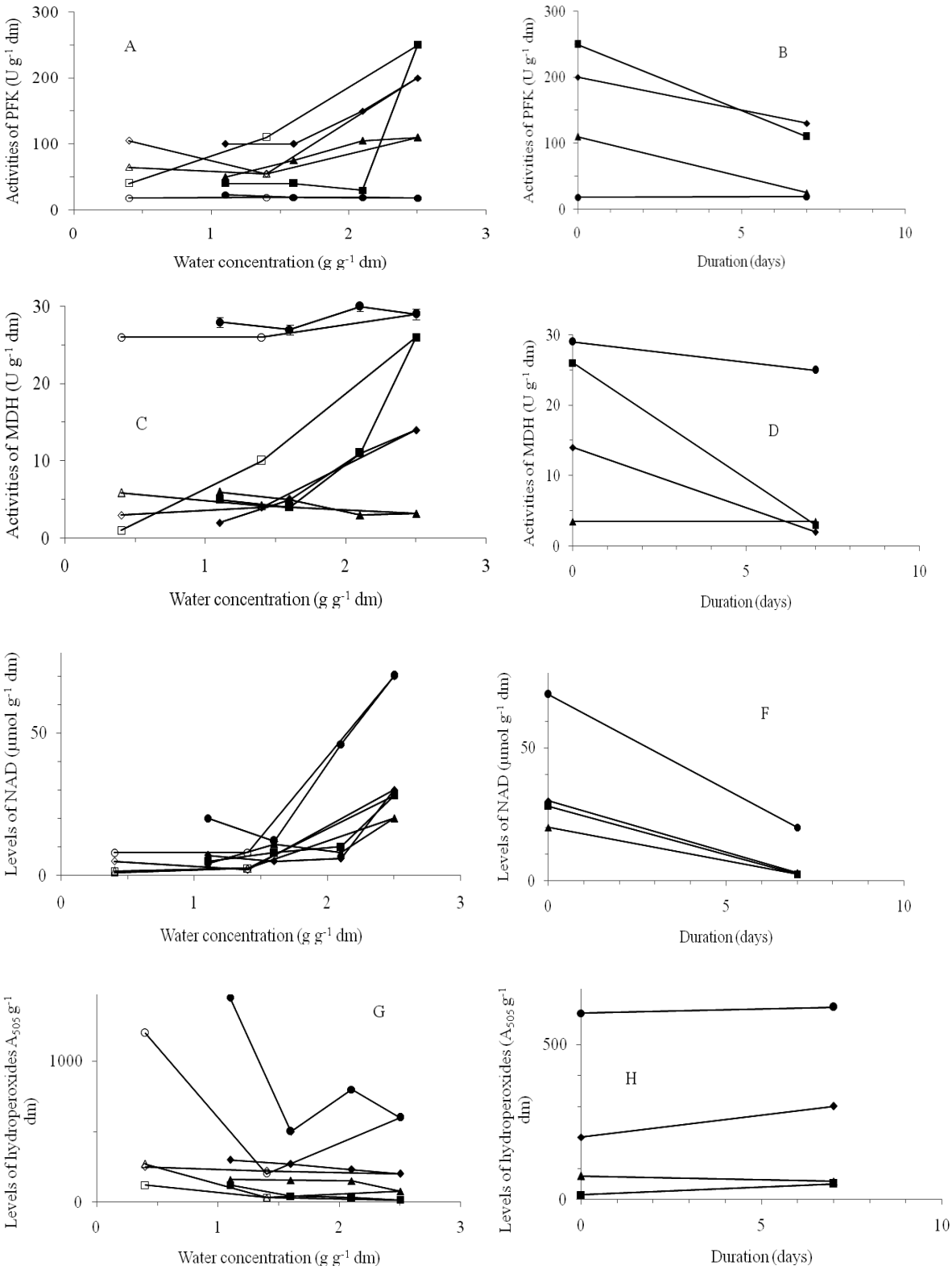

Figure 3. Activities of phosphofructokinase and malate dehydraogenase and levels of nicotinamide adenine dinucleotide and hydroperoxides in whole axes (diamonds), hypocotyls (squares), root primordia (triangles) and hypocotyls (circles) of *A. marina* during rapid (closed symbols) and slow (open symbols) drying (A, C, E and G) or hydrated storage (B, D, F and H). (1 U of PFK will convert 1 µmol of fructose-6-phosphate and ATP to fructose-1,6-bisphosphate and ADP per minute at pH 8.0 at 30 °C and 1 U of MDH will convert 1 µmol of oxaloacetate and NADH to malate and NAD per minute at pH 7.5 at 25°C).

Table 1. Statistical analysis of results.

Test	F -value	p-value
Phosphofructokinase		
1	F = 1.585.70	p = 0.02
2	F = 113.32	p = 0.01
Malate dehydrogenase		
1	F = 288.00	p < 0.01
2	F = 288.00	p < 0.01
Nicotinamide adenine dinucleotide 1	F = 791.50	p < 0.01
Hydroperoxide levels		
1	F = 80.50	p < 0.01
2	F = 120.20	p < 0.01
Superoxide dismutase		
1	F = 1 789.80	P = 0.02
2	F = 288.00	p = 002
Catalase 1	F = 37.00	p = 0.03
Glutathione reductase 1	F = 33.74	p < 0.01
2	F = 61.20	p < 0.01
3	F = 63.10	p = 0.02
4	F = 27.10	p = 0.01
5	F = 25.70	p = 0.01
Ascorbic acid 1	F = 29.00	p = 0.03

during drying and wet storage (Figures 4G and H).

DISCUSSION

The water content of axes of *A. marina* remained constant during wet storage as the surrounding air is fully hydrated and there is no direct contact between the axes and the water. Hence, water loss from the seed to the surrounding air and water uptake by the axes are in equilibrium. In agreement with studies on other species (Berjak et al., 1984; Farrant et al., 1985; Pritchard, 1991; Ntuli et al., 2011), germination and tetrazolium tests revealed that rapid drying of axes of white mangrove seeds markedly decreased the water content at which abrupt loss of viability occurred in comparison with slow dehydration.

Loss of viability occurred at high (0.8 g g^{-1} dm) water contents during desiccation of axes of *A. marina*. This observation indicates that physical damage underlay loss of viability during desiccation in *A. marina* axes. In wet storage, survival began to decline after a week suggesting that ageing may have contributed to viability loss during slow drying.

The relationship between electrolyte leakage and water concentration during drying did not show the typical pattern in which there is constant leakage to a critical water content, at which point a sudden increase is observed. Rather, there was a gradual increase in the degree of leakage as dehydration proceeded and Ntuli et al. (2011) showed a similar pattern during drying of whole seeds of *Ekebergia capensis* and excised axes of *Quercus robur*, respectively. Nevertheless, less electrolyte leakage was observed during rapid than slow dehydration. It is suggested that less membrane damage occurred during rapid desiccation because of the limited period of exposure to stress compared with slow drying.

The activities of PFK and MDH decreased upon drying and wet storage of axes, hypocotyls, root primordia and plumules of *A. marina*. This observation is in agreement with previous findings where PFK and MDH activities were reported to be adversely affected by desiccation (Leprince et al., 1993a; Côme and Corbineau, 1996; Song et al., 2009; Ntuli et al., 2011).

The levels of NAD also declined during dehydration and hydrated storage in all four tissues. This reduction is

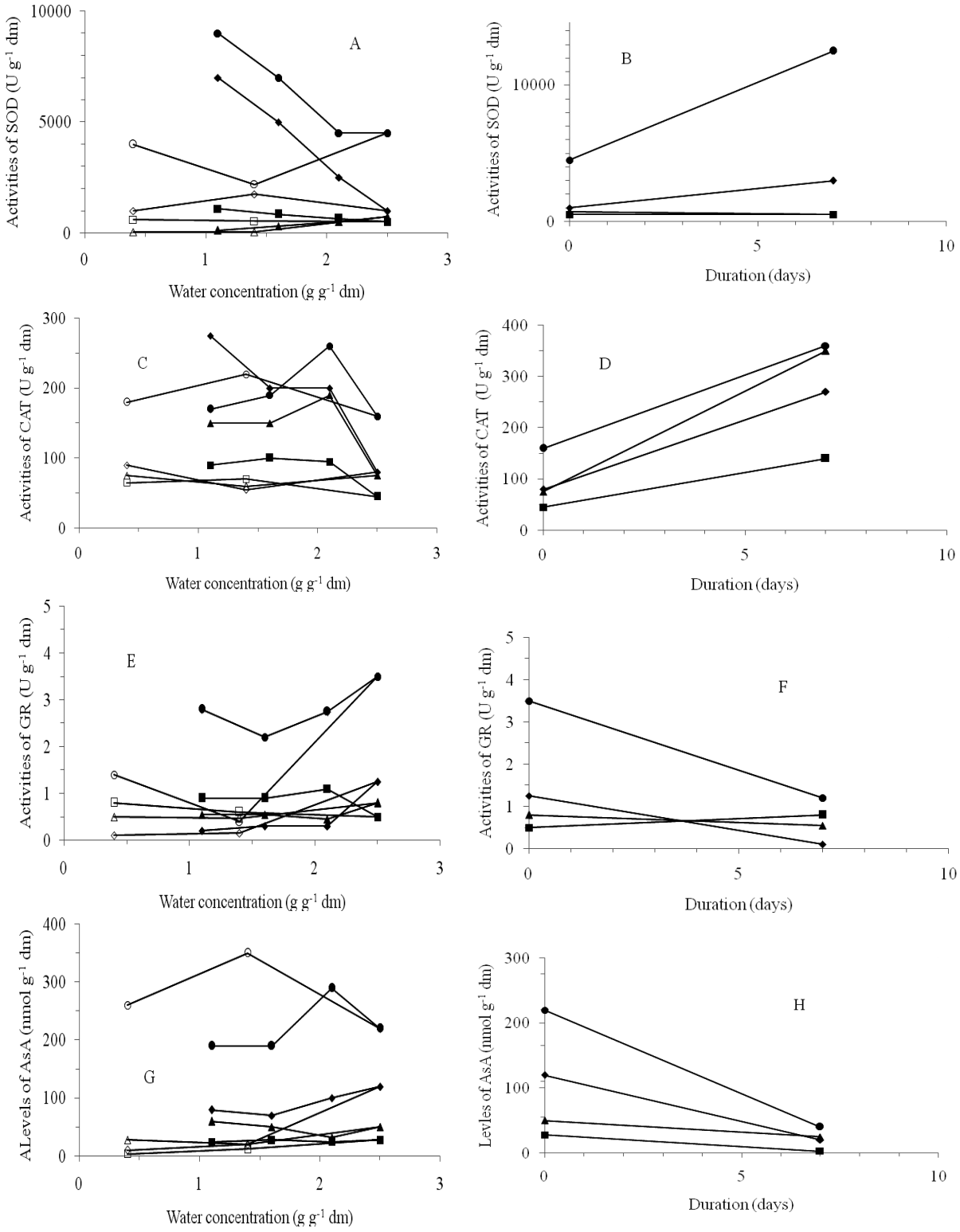

Figure 4. Activities of superoxide dismutase, catalase and glutathione reductase and levels of ascorbate in whole axes (diamonds), hypocotyls (squares), root primordia (triangles) and hypocotyls (circles) of *A. marina* during rapid (closed symbols) and slow (open symbols) desiccation (A, C, E and G) or moist storage (B, D, F and H). (1 U of SOD will inhibit the rate of reduction of cytochrome *c* by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25 °C, 1 U of CAT will decompose 1 μmol of hydrogen peroxide (H_2O_2) per minute at pH 7.0 at 25 °C, while the concentration of H_2O_2 falls from 10.3 to 9.2 mM and 1 U of GR will reduce 1 μmol of oxidized glutathione per minute at pH 7.6 at 25 °C).

attributed to the impairment of mitochondrial electron transport chain at the NADH dehydrogenases of NADH-coenzyme Q reductase (complex I) and NADH-cytochrome *c* reductase (complex IV) and the alternative oxidase (Leprince et al., 1993a; 1994; 1995; 2000; Côme and Corbineau, 1996; Leprince and Hoekstra, 1998; Song et al., 2009).

There was an increase in hydroperoxide levels during desiccation and moist storage in all tissues. This event is in concurrence with those in previous studies (for example, Leprince et al., 1990; Song et al., 2004; Huang et al., 2009; Ntuli et al., 2011).

It appears that the patterns of physiological and biochemical response of the free radical processing systems in desiccation-sensitive seeds to oxidative stress differ among both tissues and species, as in germinating orthodox seeds. For instance, the defence against oxidative attack on axial tissue of *Quercus robur* was largely dependent on antioxidants whereas it was predominantly enzymic in cotyledons (Hendry et al., 1992). Moreover, the activities of SOD and GR in axes decreased during desiccation (Hendry et al., 1992). In contrast, SOD and GR activity increased in the cotyledons upon drying. Furthermore, there was a decrease in the levels of α-tocopherol in axes during dehydration compared with an increase in the cotyledons (Hendry et al., 1992). However, the activity of SOD increased significantly during drying in *Shorea robusta* seeds (Chaitanya and Naithani, 1994) but there was a rapid decrease in activities of SOD and peroxidases in *Theobroma cacoa* axes corresponding to loss of viability (Li and Sun, 1999). Additionally, an increase in both the amount of tocopherol and activity of SOD was observed in the plumule of axes of *Avicennia marina* following dehydration (Greggains et al., 2001).

The activities of SOD and CAT increased during desiccation and wet storage of *A. marina* axes, hypocotyls, root primordia and plumules in the present study. In contrast, the GR activities and levels of AsA decreased upon drying and hydrated storage.

In summary, respiratory enzymes, PFK, MDH and the NADH dehydrogenases of the electron transport chain of axes of *A. marina* showed sensitivity to desiccation and wet storage. These data are consistent with the view that these events led to metabolic imbalance which, in turn, resulted in more leakage of electrons than normal from the mitochondrial electron transport chain. The increased free radical activity caused the enhanced formation of hydroperoxides during lipid peroxidation. In addition, desiccation and wet storage impaired the efficiency of the free radical processing systems. This situation is less prevalent during rapid drying than slow dehydration and hydrated storage.

The results of the present study support the hypothesis that rapid desiccation lowers the critical and lethal water concentrations for the survival of desiccation-sensitive seeds. Furthermore, they show that such a phenomenon arises as a consequence of lesser adverse effects upon metabolism and free radical processing systems when drying is more rapid. However, viability loss in axes of *A. marina* is underlain by physical rather than metabolic damage as indicated by the high water contents at which it occurred.

In conclusion, the activities and levels of all the enzymes and compounds studied, with the exception of PFK, were the highest in the plumules. However, the responses were remarkably qualitatively similar in all tissues. It is possible that the plumule, despite its relatively small volume and mass, plays a disproportionately important role in the loss of viability in axes of *A. marina*.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Antioxidant activities in extracts of selected indigenous vegetables from Kenya and Malawi

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Antioxidant activities and phytochemical compounds of ethanol and hot water extracts of 7 selected indigenous vegetable species from Malawi and Kenya were Spectophotometrically determined and evaluated. Their effectiveness were also evaluated by their EC_{50} values through interpolation from linear regression analysis of their respective data. Generally, ethanolic extracts portrayed high quantities of total phenol, carotenoids and lycopene while hot water extracts showed high ascorbic acid. The highest total phenol (475.88±0.02 mg/g) and lycopene (0.13±0.02 mg/g) were detected in the ethanol extracts of *I. batatas* and *C. gynandria*, respectively. In the hot water extracts, the highest ascorbic acid (2.59±0.06 mg/g) and flavonoids (156.43±0.02 mg/g) were from M. *esculenta.* Dose-dependent antioxidant activities of the extracts were observed. Based on the EC_{50} values (mg/ml), the hot water extracts were significantly ($p<0.05$) more effective in all antioxidant activities assayed (DPPH, hydroxyl, superoxide anion radicals and reducing power) than ethanol extracts. It was observed that a single vegetable species did not posses all sorts of antioxidant phytochemical compounds in significant quantities and hence not effective in scavenging all different radicals. A combinatory intake of these vegetables species in sufficient concentrations should thus be recommended to enhance an optimal antioxidant capacity in the body.

Key words: Antioxidants, free radicals, health benefits, indigenous vegetables.

INTRODUCTION

Humans are constantly exposed to reactive oxygen species (ROS) produced by natural phenomena such as ultraviolet light or by anthropogenic activities. Excessive productions of these ROS than the body antioxidant system effectively terminate or retard them can negatively have impact on health by causing several oxidative stress related diseases including cancer, hypertension, heart diseases and diabetes (Young and Woodside, 2001). The human body counteracts these diseases by producing antioxidants which are either naturally produced *in situ*, or externally supplied through foods or supplements. However, the available synthetic antioxidant supplements such as butylated hydroxyanisole, butylated hydroxytoluene and gallic acid esters have

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Table 1. Plant samples used for the study.

Kenya		Malawi	
Botanical name	**Vernacular/common name**	**Botanical name**	**Vernacular/common name**
Urtica ferox	Thabai, Hatha/Stinging nettle	*Ipomoea batatas*	Kholowa/Sweet potato leaves
Corchorus olitorius	Mlenda, Jute/ Saluyot	*Amaranthus spinosus*	Bonongwe/Green Amaranth leaves
Cleome gynandra	Sagati/Spiderplant	*Manihot esculenta*	Chigwada/Cassava leaves
Solanum psedocapsicum	Monagu/Osuga/Black night shade		
Brassica oleracea (exotic vegetable)			

been suspected to cause or prompt negative health effects and observed to poses moderate antioxidant activities (Jeetendra et al., 2010). There is now a growing interest to substitute synthetic antioxidants with naturally occurring antioxidants from plant sources.

Most *in vitro* studies on plants have strongly supported the idea that plants constituent antioxidant activities capable of exerting protective effects against oxidative stress in biological systems (Rahmat et al., 2014; Kumar et al., 2010; Shimada et al., 2004). Apart from traditionally used natural antioxidants from tea, wine, and spices (Cao et al., 2012; Amro et al., 2002; Moure et al., 2001), Tomatoes, watermelons, guavas, papayas, apricots, pink grapefruits, blood oranges have also been observed to contain excellent antioxidant properties and high quantities of Carotenoids and lycopene (Johnson, 2002).

Strong antioxidant properties and high quantities of phytochemical compounds were also observed in Cap and Stipe from chicken drumstick mushroom species of *Coprinus* (Bo et al., 2010) and *Grifola frondosa*, *Morchella esculenta* and *Termitomyces albuminosus* mycelia (Mau et al., 2004). Similarly, indigenous vegetables from East-India and from West Africa have also been reported to constitute high levels of antioxidant components and activities (Odukoya et al., 2007; Gyingiri et al., 2012; Handique and Boruah, 2012)

Although many plant species have been investigated in search for novel antioxidants so far, fewer studies have been conducted on the edible green leafy indigenous vegetables of Sub-Saharan Africa and there is a high demand for a specific scientific data on their antioxidant potential. The present study qualitatively and quantitatively analyzed and determined the antioxidant phytochemicals compounds and evaluated antioxidant activities of seven indigenous vegetable species consumed in Kenya and Malawi summarized in Table 1 using spectroscopic methods. In the longer term, extracts of the vegetable species (or their active constituents) identified as having high levels of antioxidant activities *in vitro* may be of value in the design of further *in vivo* studies to unravel novel treatment strategies for disorders associated with ROS.

MATERIALS AND METHODS

Reagents, chemicals and standards

L-ascorbic acid, Tannic acids, Folin-ciocalteu's phenol, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) were all purchased from Sigma-Aldrich, Germany. Sodium carbonate, aluminium chloride, 2, 6-Dichlorophenolindophenol and methanol were purchased from BDH Poole, England. All the chemicals used were of analytical grade. Deionized water was used throughout the experiment. Jenway 6405 UV/Visible Spectrophotometer by Buch Scientific Inc.USA was used for analysis.

Plant materials

Fresh leafy vegetable samples were bought from the local markets in Lilongwe, Malawi and Nairobi, Kenya and transported while still fresh in Cold chain box of between 2 to 6°C to University of Nairobi, Department of Biochemistry laboratory and kept at -80°C. *Brassica oleracea* was also bought from Kenya local market and studied for comparison because it is an exotic vegetable and it is widely consumed by most communities. The vegetables species used in the study are shown in Table 1.

Sample preparations and extraction

Samples were cleaned with deionized water and the leafy edible portions were chopped into very small tiny pieces prior to extraction. Two methods of extraction were deployed; hot water and ethanol (95% pure) extraction according to Bo et al. (2010). The hot water extracts were freeze-dried while the ethanol extracts rotary evaporated at 40°C to dryness. The dried extracts were used directly for analyses of antioxidant components or redissolved in water or ethanol to a concentration of 50 mg/ml and stored at 4°C for further analysis.

Determination of antioxidant phytochemicals

Total phenolic content

The total phenolic content of extracts was determined according to Barros et al. (2008) by Folin-Ciocalteu spectrophotometric method**.** Extract samples (1 ml) was mixed with equal volume of Folin and Ciocalteu's phenol reagent. After 3 min, 1 ml of saturated sodium carbonate solution was added to the mixture and adjusted to 10 ml with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm. Tannic acid was used to construct the standard curve (0.01-0.4 mM). Estimations of

the phenolic compounds were carried out in triplicate. The results were presented as mean of three measurements and expressed as mg of tannic acid equivalents (TAEs) per g of each extract.

Total flavonoids content

Total flavonoid content was determined by the formation of a complex of aluminum flavonoid using the methodology of Oyedemi et al. (2010). Extracts solution 1 ml was diluted with 4.3 ml of 80% aqueous ethanol and to the test tubes 0.1 ml of 10% aluminium nitrate was added followed by 0.1 ml of 1 M aqueous potassium acetate. After 40 min at room temperature, the absorbance was determined spectrophotometrically at 415 nm. Total flavonoids concentration was calculated using quercetin as the standard. Absorbance = 0.002108 µg quercetin - 0.01089. The results are presented as mean of three measurements and expressed as mg of quercetin per g of each extract.

Ascorbic acid determination

Ascorbic acid was determined from the extracts using the 2, 6-dichloro-phenolindophenol (Barros et al., 2008). The dried extract (100 mg) was dissolved in metaphosphoric acid (1%, 10 ml) for 45 min at room temperature and filtered through Whatman No. 4 filter paper. The filtrate (1 ml) was mixed with 2, 6-dichlorophenolindophenol (9 ml) and the absorbance was then measured within 30 s at 515 nm against a blank. Content of ascorbic acid were calculated on the basis of the calibration curve of authentic L-ascorbic acid (0.020-0.12 mg/ml; Y = 3.4127X - 0.0072 and results are presented as mean of three measurements, expressed as mg of ascorbic acid/g of extract.

β- Carotene and lycopene determination

β- Carotene and lycopene were determined according to the acetone-hexane mixture method of Nagata and Yamashita (1992) with some modifications. The dried extract (50 mg) was vigorously shaken with acetone-hexane mixture (4:6, 5 ml) for 1 min and filtered through Whatman No. 4 filter paper. The absorbance of the filtrate was then measured at λ = 453, 505 and 663 nm. Contents of β-carotene and lycopene were calculated according to the following equations: Lycopene (mg/100 ml) = - 0.0458 A663 + 0.372 A505 - 0.0806 A453; β-carotene (mg/100 ml) = 0.216 A663 - 0.304 A505 + 0.452 A453. The results are presented as mean of three measurements and expressed as mg of carotenoid/g of extract.

Antioxidant activities determination

DPPH Radical scavenging activity

The antioxidant activity of the extracts was measured on the basis of the scavenging activity of the stable 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) free radical according to the spectrophotometric method of Mensor et al. (2001). 1 ml of a 0.3 mM DPPH methanol solution was added to 2.5 ml solution of the extract or standards and allowed to react at room temperature for 30 min. The absorbance of the resulting mixture was then measured at 518 nm and converted to percentage antioxidant activity.

Hydroxyl radical scavenging activity

Scavenging ability on hydroxyl radicals was determined according to the method of Smirnoff and Cumbers (1989) with some modifications. Hydroxyl radicals from the Fenton reaction reacted with salicylate to form a colored material (2,3-dihydroxybenzoate). The hydroxyl radicals scavenging activity of an extract was assayed by the colour change of reaction system. 1 ml of the extract was mixed with 1 ml of $FeSO_4$ (9 mM) and 1 ml of salicylic acid (9 mM) in 95% ethanol. The reaction was initiated by the addition of 1 ml of H_2O_2 (8.8 mM). After 30 min incubation at 37°C, the absorbance of the mixture was determined at 510 nm against a blank and converted to percentage antioxidant activity.

Superoxide anion radical scavenging activity

The ability of extracts to scavenge superoxide radicals was measured according to Martin et al. (1987) spectrophotometric method where the superoxide anion radicals were generated from auto-oxidation of hematoxilin and was detected by an increase in absorbance at 560 nm. To a mixture (phosphate buffer 0.1M, pH 7.4, EDTA 0.1M and hematoxilin 50 µM), 1 ml of extract was added and incubated at 25°C for 10 min. Inhibition of auto oxidation of hematoxilin by boiled and ethanol extracts over the control was then measured.

Reducing power assay

The Oyaizu (1986) method was followed with some modifications to measure the power of extracts to reduce ferricyanide to ferrocyanide. A 2.0 ml of the extract was added to 2.0 ml of phosphate buffer (0.2 M, pH 6.6) and 2.0 ml of potassium ferricyanide (10 mg/ml), and the mixture incubated at 50°C for 20 min. 1 ml of Trichloroacetic acid (50 mg/ml) was then added, and the mixture centrifuged at 4,000 rpm for 10 min. The upper layer (2.5 ml) was then gently mixed with deionised water (2.5 ml) and 0.5 ml ferric chloride (1 mg/ml) and the absorbance measured at 700 nm against a blank.

Determination of the EC_{50}

The EC_{50} was the effective concentration at which the DPPH radicals, hydroxyl radicals or superoxide radicals were scavenged by 50%, and absorbance was 0.5 for reducing power. This was obtained by interpolation from linear regression analysis of their respective data and results were normalized and expressed as EC_{50} values (mg/ ml) for comparison. The lower the EC_{50} value, the more efficient the sample was.

Data analysis

The experimental data were subjected to an analysis of variance (ANOVA) for a completely random design to determine the least significant difference at the level of 0.05.

RESULTS AND DISCUSSION

Antioxidant components

Quantities of total ascorbic acid, flavonoids, phenol, β carotenoids and lycopene detected are shown in Table 2. Significantly higher ($p<0.05$) quantities of total phenol, carotenoids and lycopene were detected in extracts of ethanol while ascorbic acid was detected in hot water extracts. No significant difference in total flavonoids content between hot water and ethanolic extracts was observed. The present findings of higher content of ascorbic acid obtained in hot water extracts contradict

Table 2. Contents of ascorbic acid, total flavonoids, total phenols, carotenoids and lycopenes in various sample extracts.

Extract	Vegetable specie	Antioxidant component (mg/g of extract)				
		Ascorbic acid[a]	Flavonoids[b]	Phenols[c]	βCarotenoids[d]	Lycopenes[d]
Ethanolic	*I. batatas*	1.93 ± 0.01	118.48 ± 0.06	475.88 ± 0.02	0.32 ± 0.08	0.08 ± 0.02
	A. spinosus	2.20 ± 0.02	99.31 ± 0.02	49.73 ± 0.03	0.36 ± 0.03	0.07 ± 0.03
	M. esculenta	0.70 ± 0.04	101.68 ± 0.03	246.77 ± 0.07	0.42 ± 0.03	0.07 ± 0.01
	Urtica ferox	2.17 ± 0.03	44.41 ± 0.04	41.11 ± 0.03	0.35 ± 0.02	0.04 ± 0.01
	S. *psedocapsicum*	2.25 ± 0.05	21.74 ± 0.06	43.06 ± 0.04	0.15 ± 0.03	0.04 ± 0.002
	C. olitorius	1.04 ± 0.03	105.54 ± 0.03	158.59 ± 0.02	0.57 ± 0.04	0.04 ± 0.003
	C. gynandra	1.27 ± 0.02	13.96 ± 0.04	331.04 ± 0.01	0.71 ± 0.02	0.13 ± 0.02
	B. oleracea	2.40 ± 0.03	1.31 ± 0.03	11.24 ± 0.05	0.78 ± 0.02	n.d
Hot water	*I. batatas* tops	2.57 ± 0.05	149.69 ± 0.06	54.86 ± 0.07	0.02 ± 0.001	0.05 ± 0.001
	A. spinosus	1.96 ± 0.01	7.79 ± 0.02	23.38 ± 0.02	0.04 ± 0.01	0.02 ± 0.001
	M. esculenta	2.59 ± 0.06	156.43 ± 0.02	56.52 ± 0.03	0.06 ± 0.02	0.05 ± 0.002
	Urtica ferox	2.50 ± 0.02	57.25 ± 0.03	14.31 ± 0.04	0.01 ± 0.001	n.d
	S. *psedocapsicum*	2.49 ± 0.03	141.31 ± 0.04	20.88 ± 0.04	0.08 ± 0.02	0.04 ± 0.01
	C. olitorius	2.43 ± 0.04	102.60 ± 0.04	51.54 ± 0.03	0.02 ± 0.01	0.01 ± 0.005
	C. gynandra	2.48 ± 0.07	119.87 ± 0.02	11.33 ± 0.02	0.06 ± 0.01	0.01 ± 0.003
	B. oleracea	2.56 ± 0.02	6.55 ± 0.001	8.40 ± 0.03	0.05 ± 0.02	0.01 ± 0.002

n.d=not detected or <0.01, each value is expressed as mean±SD (n=3); [a] mg ascorbic acid/g of extract; [b] mg of quercetin/g of extract; [c] mg of TAEs/g of extract; [d] mg of β carotenoids or lycopene/g of extract.

with the findings of Oboh et al. (2008) and, Adefegha and Oboh (2009) on tropical vegetables where high quantities of ascorbic acid were obtained in ethanol extracts. The observed variation between the current and the two studies might be due to the extraction methodology such that while they used distilled water at room temperature for extraction, boiling distilled water was used in this study which resulted in better dissolution of the ascorbic acid. Nevertheless, the best solvent for the extraction of compounds in plant food depends very much on the variety of constituents in the food matrix. It is therefore difficult to develop a general protocol for extraction of different compounds from various matrices. Ascorbic acid content in hot water extracts ranged from 1.96±0.01 mg/g (*A. spinosus)* to 2.59 ±0.06 mg/g (*M. esculenta)* and in ethanol extracts was between 0.70±0.04 mg/g (*M. esculenta*) to 2.40±0.03 mg/g (*B. oleracea*). The highest ascorbic acid content obtained in hot water extracts of *M. esculenta* agrees with the findings of Gyingiri et al. (2012) who also found relatively high quantities of ascorbic acid on the same vegetable species of Ghana and suggests the potential of the vegetable as a good source of ascorbic acid.

Although we found that there was no significant difference in the quantity of flavonoids between the extract, Sumazian et al. *(*2010) working on Malaysian vegetables found that flavonoids responded better in aqueous boiled extracts. Our present findings may suggest that flavornoids are relatively stable compounds and can be easily retained with either of the two extraction methods.

The range for total phenol content was between 8.40±0.03 *(B. oleracea)* and 56.52±0.03 (*M. esculenta)* for hot water and between 11.24±0.05 (*B. oleracea*) and 475.88±0.02 *(I. batatas)* for ethanolic extracts, respectively. This suggest that the studied indigeneous vegetables contain high quantities of phenol than the exotic *B. oleracea.* The current study further found that ethanol extracts had high phenol than hot water extracts. Phenolic acids have repeatedly been implicated as natural antioxidants in fruits and vegetables known to be effective antioxidants due to their hydroxyl groups (Boskou, 2006).

Our study reported *B*-carotenoid content ranging from 0.01±0.001 *(U. ferox)* to 0.08±0.02 (S*. psedocapsicum)* and 0.15±0.03 (S*. psedocapsicum)* to 0.78±0.02 (*B. oleracea*) for hot water and ethanol extracts, respectively. The highest quantities of lycopene detected were 0.05±0.002 (*M. esculenta)* and 0.13±0.02 *(C. gynandra)*. The higher quantity of both carotenoids and lycopene in ethanolic extracts than hot water extracts suggest that ethanol is good extraction solvent for these compounds as has been also observed in Rao and Rao (2007).

Antioxidant properties

Hot water extracts had high DPPH scavenging abilities than ethanolic extracts ($p<0.05$). The DPPH radical scavenging ability for hot water extracts was between 51.05% (*B. oleracea)* and 96.74% (*I. batatas)* (Figure 1a) while for ethanolic extracts was from 31.03% (*B. oleracea)* to 95.40% (*C. gynandra)* (Figure 1b) at 20

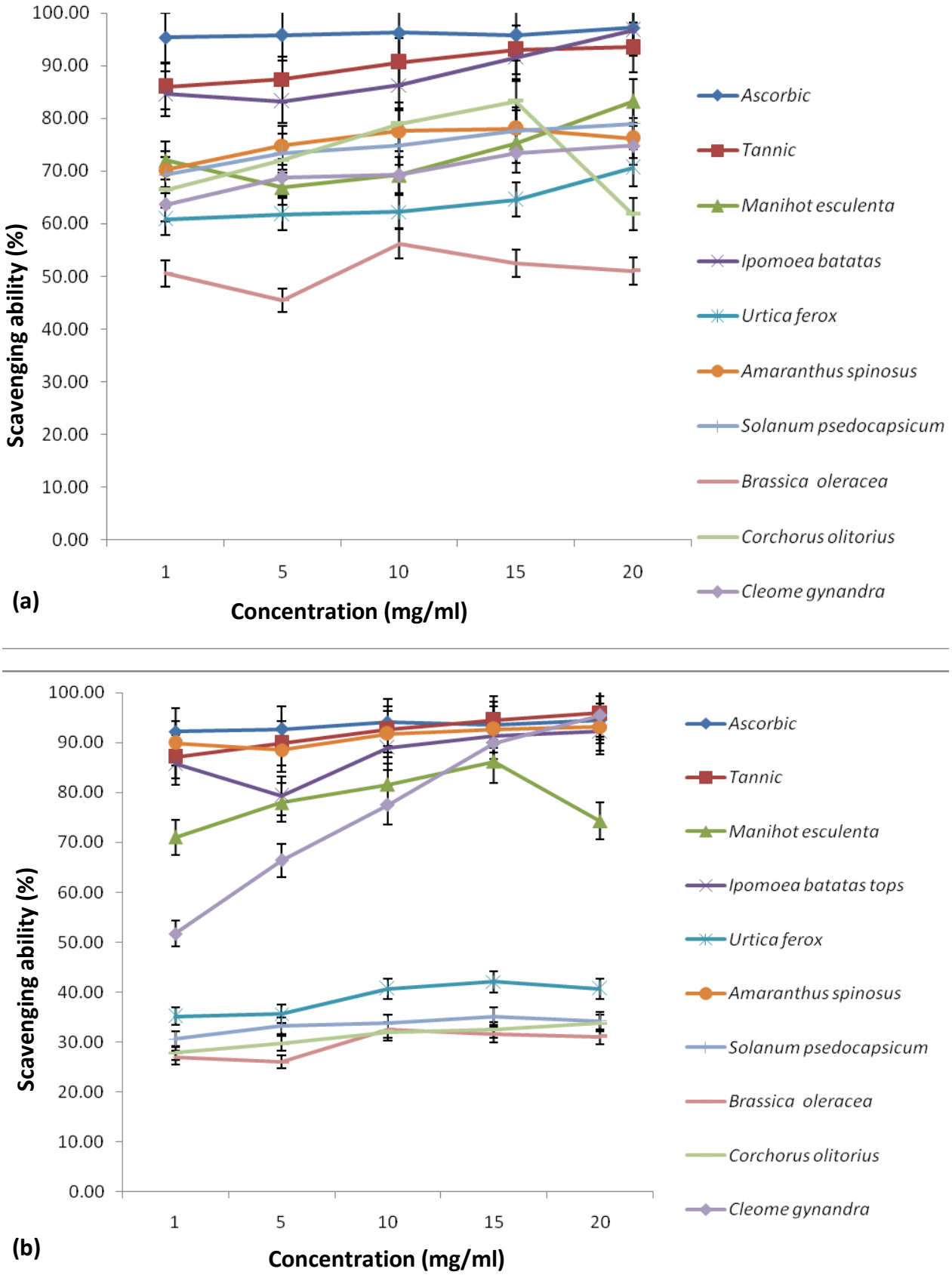

Figure 1. Scavenging ability of **(a)** hot water extracts **(b)** ethanolic extracts of various samples on DPPH radicals. Each value is expressed as mean ±SD (n= 3).

mg/ml. This finding highlights the value of indigenous vegetables against the exotic commercial vegetables like *B. oleracea* in scavenging DPPH radicals.

Hydroxyl radicals are the major active oxygen species that cause lipid oxidation and immense biological damage (Kumar et al., 2008). They can react with lipids, polypeptides, saccharides, nucleotides, and organic acids, especially thiamine and guanosine, thereby causing cell damage (Jiao et al., 2005). Hydroxyl radical scavenging ability was from 79.22% (*C. gynandra)* to 95.01% (*B. oleracea)* for hot water extracts (Figure 2a) while for ethanol extracts was between 64.38% (*C. gynandra)* and 80.52% (*M. esculenta)* (Figure 2b). No significant difference ($p<0.05$) in hydroxyl radicals scavenging ability between hot water and ethanol extracts was observed.

The hot water extracts had high abilities in scavenging superoxide anion radicals than ethanol extracts ($p<0.05$). The highest superoxide radicals scavenging ability for hot water extracts was 91.29% (S*. psedocapsicum)* at 15 mg/ml with a gradually decrease to 81.79% at 20 mg/ml and the lowest ability was 36.66% *(C. gynandra)* at 20 mg/ml (Figure 3a). For the ethanol extracts, superoxide anion scavenging abilities was between 8.48% *(A. spinosus)* and 81.07% *(C. gynandra)* at 20mg/ml (Figure 3b). The highest superoxide anion scavenging abilities observed in hot water extracts of S*. psedocapsicum* and ethanol extracts of *C. gynandra* might be due to their high content of phenol, carotenoids and lycopene detected in these species.

The reducing power ranged from 0.33 *(C. gynandra)* to 2.31 (*M. esculenta)* for hot water extracts (Figure 4a) and 0.32 *(S. psedocapsicum)* to 2.76 (*A. spinosus)* (Figure 4b) at 20 mg/ml. No significant differences ($p<0.05$) in reducing power between hot water and ethanol extracts was detected. The reducing power of both extracts increased with increasing concentration of the extract, indicating that some compounds in the extracts were electron donors and could also react with free radicals to convert them into more stable products and to terminate radical chain reactions.

The EC_{50} values in antioxidant properties

The EC_{50} values in antioxidant properties assayed herein are summarized in Table 3 and the results were normalized and expressed as EC_{50} values (mg/mL) for comparison. Effectiveness of antioxidant properties inversely correlated with their EC_{50} values.

Generally, the hot water extracts were significantly ($p<0.05$) more effective in all antioxidant properties assayed than ethanol extracts. This finding contradicts with (Yang et al., 2006; Jan et al., 2011) who found ethanol extracts to be more effective than aqueous extracts. This contradiction might be due to the fact while they used distilled water at room temperature; our extracts were boiled with distilled water to completely dissolve antioxidant compounds in the vegetable species. It was also observed that the ascorbic acid and tannic acid (standards) used were more effective in most antioxidant properties assayed than the vegetable species. This further supports the claims of Olajire and Azeez (2011), Smith and Eyzaguirre (2007), and Sumazian et al. (2010) but contradicts with Pourmorad et al. (2006).

With regard to DPPH radicals scavenging ability, extracts of *A. spinosus* were the most effective (0.56 mg/ml) among the ethanolic extracts while extracts of *I. batatas* were the most effective (0.59 mg/ml) among the hot water extracts.

Interestingly, the extracts of *B. oleracea* were the most effective in scavenging hydroxyl radicals among both ethanolic and hot water extracts with relatively lower EC_{50} values of 0.56 and 0.73 mg/ml, respectively.

Extracts of *C. gynandra* and *M. esculanta* were the most effective (0.91 and 11.04 mg/ml) extracts in scavenging superoxide anion radicals among ethanol and hot water extracts, respectively. Among the ethanolic extracts, *A. spinosus* was the most effective (0.99 mg/ml) extract in reducing power while *C. olitorius* was the most effective (0.59 mg/ml) in reducing power among the hot water extracts.

The present EC_{50} findings agree with (Pietta, 2000; Lako et al., 2007,) on radical- specific antioxidant potentials of plants and suggests that a single vegetable species or an extract might not offer 100% efficiency in scavenging all sorts of radicals the body system might produce.

Conclusion

From the findings, the seven indigenous vegetables exhibit appreciable antioxidants activities and phytochemical compounds. Dose-dependent antioxidant activities of the extracts were observed. Based on the EC_{50} values, Hot water extracts were more effective than ethanol extracts. It was observed that a single vegetable species did not posses all sorts of antioxidant phytochemical compounds in significant quantities and hence not effective in scavenging all different radicals. A combinatory intake of these vegetables species in sufficient concentrations should thus be recommended to enhance optimal antioxidant capacity in the body. Investigations of individual compounds, their *in vivo* antioxidant activities and mechanisms are needed.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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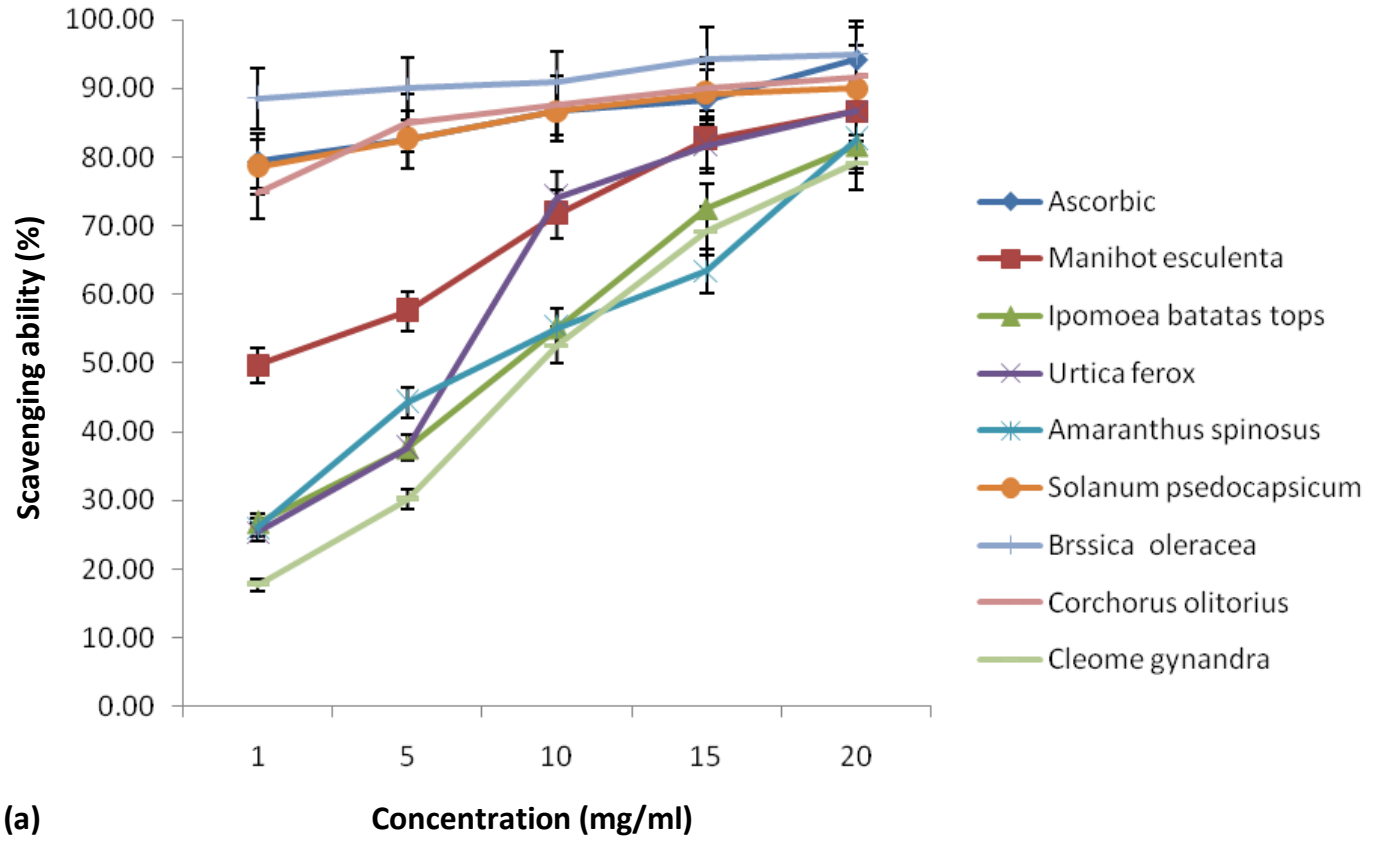
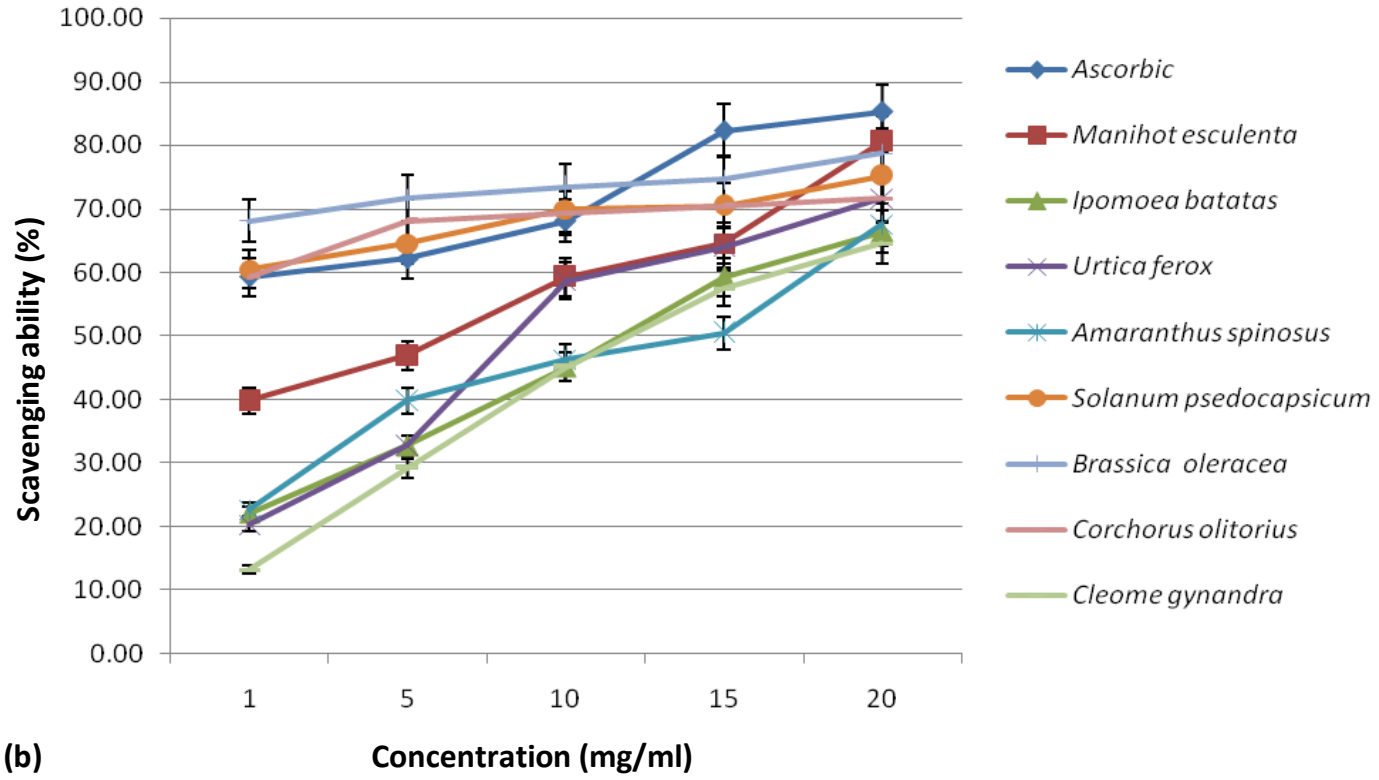
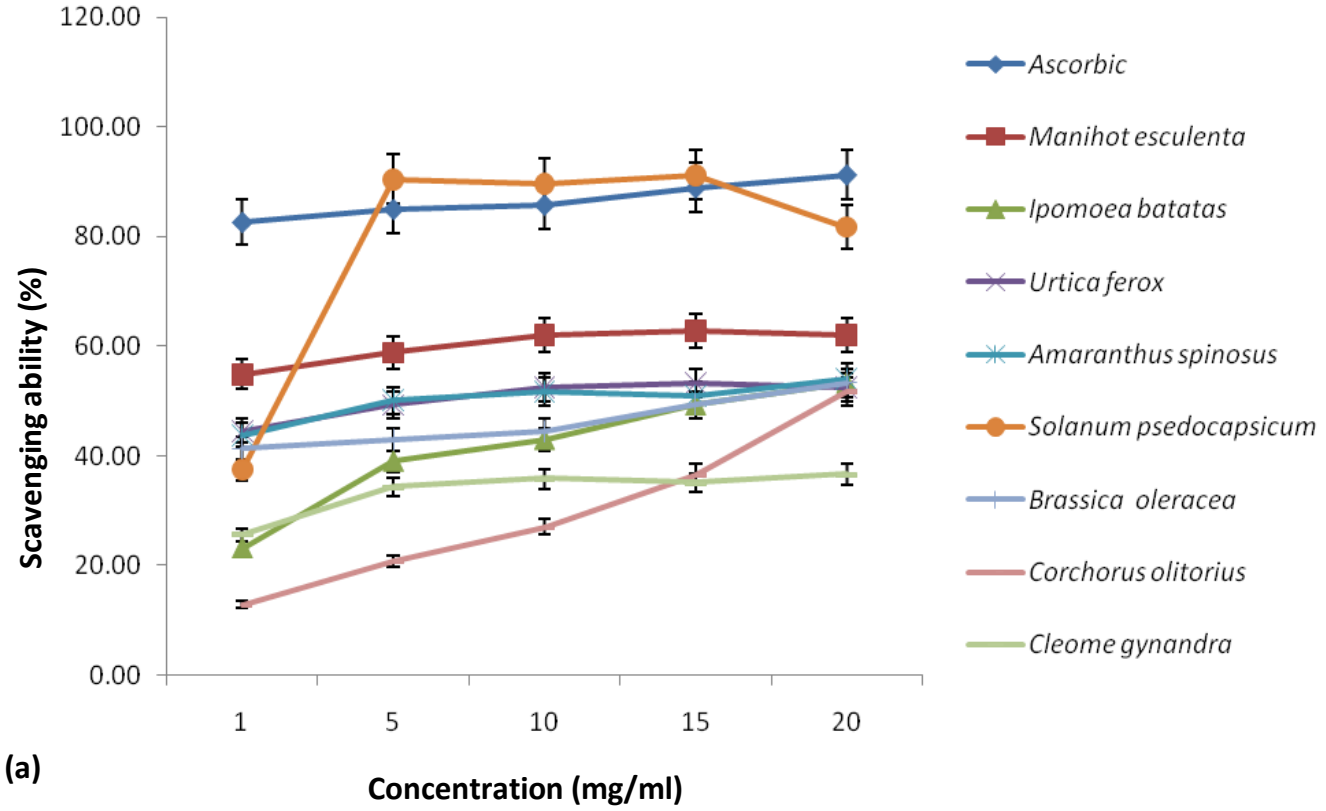


(a)

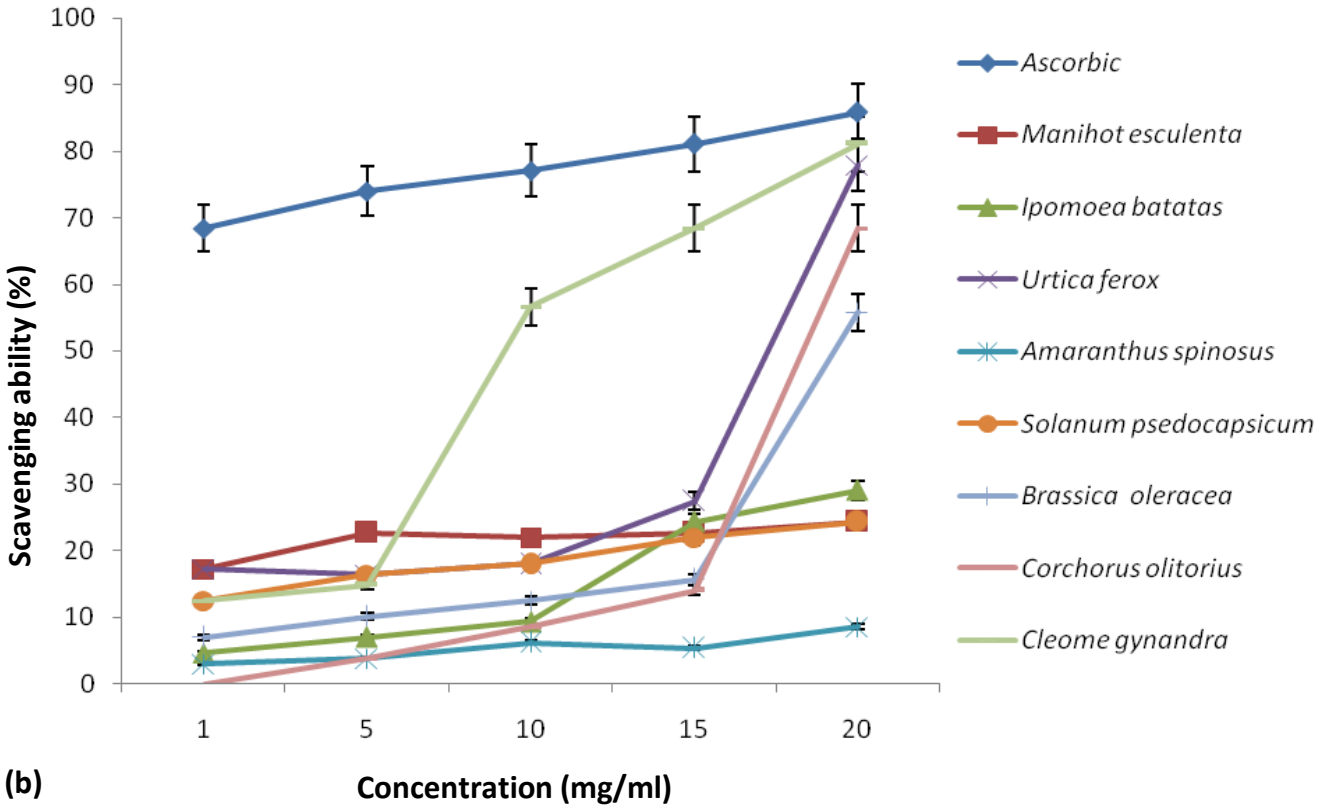


(b)

Figure 2 Scavenging ability of **(a)** hot water extracts **(b)** ethanolic extracts of various samples on hydroxyl radicals. Each value is expressed as mean ± SD (n=3).



(a)



(b)

Figure 3 Scavenging ability of **(a)** hot water extracts **(b)** ethanolic extracts of various samples on superoxide anion radicals at 25°C incubation for 10 min. Each value is expressed as mean ± SD (n=3)

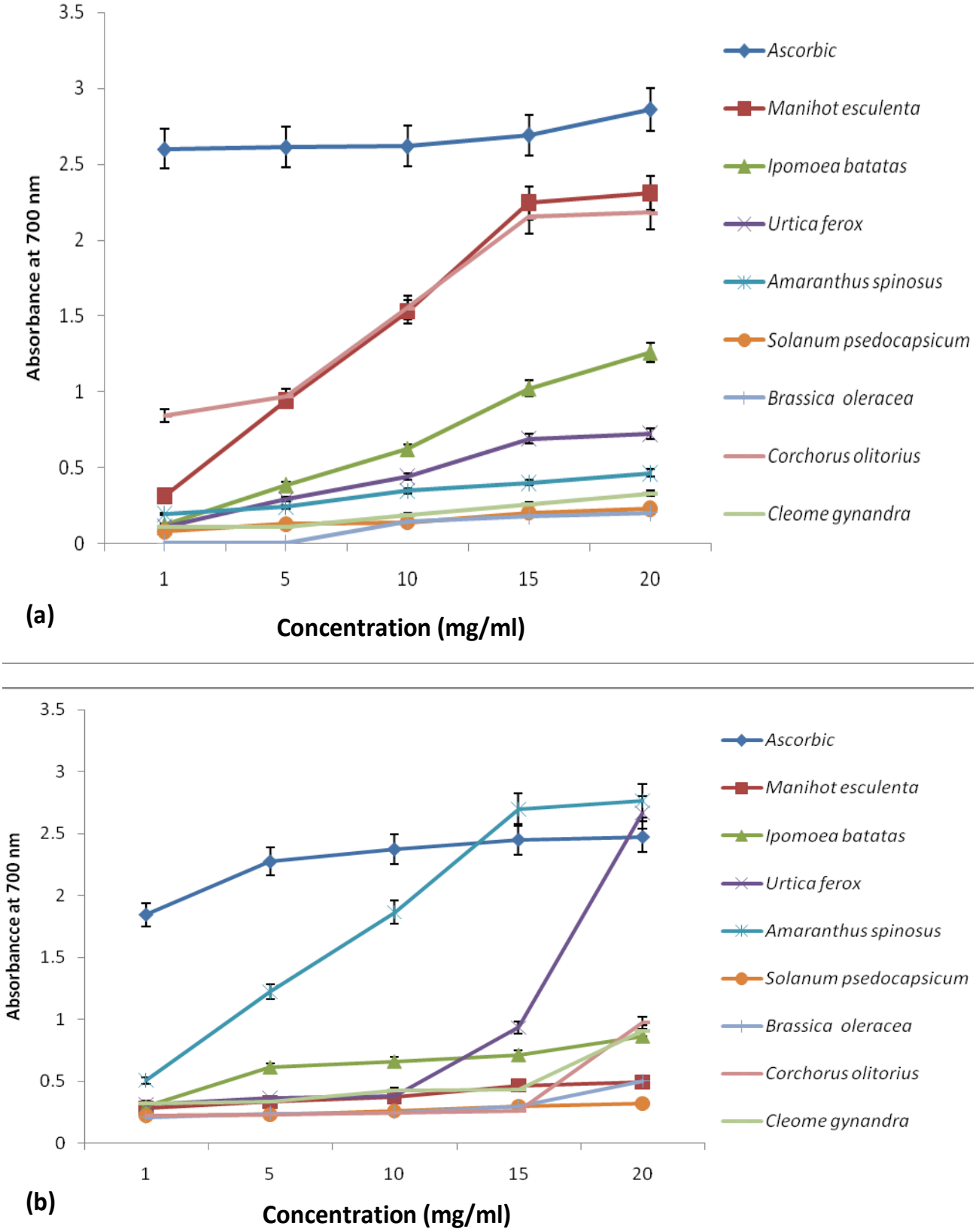


Figure 4. Reducing power of **(a)** hot water extracts **(b)** Reducing power of ethanolic extracts of various samples. Each value is expressed as mean ± SD (n=3).

Table 3. EC_{50} values of ethanolic and hot water extracts from various vegetable species in antioxidant properties.

Extract	Antioxidant attribute	EC_{50} (mg/mL)									
		Ascorbic acid	*Tannic acid*	*M. esculenta*	*I. batatas tops*	*U. ferox*	*A. spinosus*	*S. psedocapsicum*	*B. oleracea*	*C. olitorius*	*C. gynandra*
Ethanolic	1. Scavenging ability on DPPH radicals	0.54	0.57	0.70	0.58	n.e	0.56	n.e	n.e	n.e	0.97
	2. Scavenging ability on OH radicals	0.84	n.d	6.23	12.25	10.39	12.45	0.83	0.73	0.84	13.18
	3. Scavenging ability on superoxide radicals	0.73	n.d	n.e	n.e	16.83	n.e	n.e	23.88	20.19	11.04
	4. Reducing power	0.27	n.d	19.69	5.08	6.35	0.99	n.e	n.e	13.69	10.65
Hot water	1. Scavenging ability on DPPH radicals	0.52	0.58	0.69	0.59	0.82	0.71	0.72	3.45	0.75	0.79
	2. Scavenging ability on OH radicals	0.63	n.d	0.64	8.61	6.98	8.64	0.64	0.56	0.67	10.26
	3. Scavenging ability on superoxide radicals	0.61	n.d	0.91	16.03	9.12	9.93	1.34	16.04	20.53	n.e
	4. Reducing power	0.19	n.d	1.39	7.18	11.56	n.e	n.e	n.e	0.59	n.e

Each value is expressed as mean of three measurements. n.e= no effect, n.d= not done.

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