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*Full Length Research Paper*

## Rice (*Oryza sativa*) breeding strategies for grain biofortification

Fábio Luíz Checchio Mingotte\*, Lucas Tadeu Mazza Revolti, Sandra Helena Unêda-Trevisoli, Leandro Borges Lemos and Domingos Fornasieri Filho

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**Biofortification is a process in which plant species are improved in order to raise the nutritional content of the product consumed. The following are some of the main techniques that can be introduced in plant breeding programs in order to improve the nutritional quality of the food to be produced. The rice breeding for higher Fe and Zn content in grain, rice breeding for higher  $\beta$ -carotene content in the grain, rice breeding for higher folate content in the grain, techniques used for plant transformation, transformation via protoplasts, transformation via bioballistics (or biolistic), transformation via *Agrobacterium tumefaciens*, identification and *in vitro* selection of transformed tissues, concepts of gene expression and regulation, protein expression, proteomics, mutant study, *patch-clamp*, were studied. It is necessary to verify the occurrence of variability among the rice genotypes for the Fe, Zn and B complex vitamins; therefore, it will be possible to select these materials within breeding programs. The simple selection of these superior genotypes in relation to nutritional quality, even though by means of conventional breeding techniques, can bring benefits to rice consumption by the human population.**

**Key words:** Cultivated rice (*Oryza sativa*), agriculture, plant breeding, genetics, transformation, selection, gene expression, micronutrient.

### INTRODUCTION

Estimates point to an accelerated growth of the world population reaching about 9 to 10 billion people (Godfray et al., 2010; FAO, 2017). In this context, food production should accompany such demand and is considered a fundamental factor for food security in the planet. However, micronutrient deficiency in humans is mainly caused by the deficient daily diet, combined with the low

concentrations and low availability of these elements in the products consumed. Currently, deficiencies caused by the lack of iron (Fe), iodine (I), selenium (Se), vitamin A and the zinc (Zn) are the ones that cause greater concern with regard to human health. Research indicates that this deficiency affects infants, preschool children, teenagers, pregnant and women in fertile age (Zancul,

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2004; Andersson et al., 2017).

Among the strategies to reduce malnutrition in the world, food biofortification has been pointed out as one of the options of greater practical feasibility (White and Broadley, 2005; Sharma et al., 2017). In this context, significant progress has been achieved at International Rice Research Institute (IRRI) by enriching rice grain with provitamin A (b-carotene) and biofortification of iron and zinc (Andersson et al., 2017). Thus, bioavailability of these micronutrients will provide benefit to poor rice consumers, particularly in Asia to overcome the problems of vitamin A, iron and zinc deficiency in their diet (Zancul, 2004; Jena and Nissila, 2017).

Biofortification is a process in which plant species are improved in order to raise the nutritional content of the product consumed (grains, fruits, roots, tubers and leaves). This mechanism of plant breeding is the promising and relatively low cost (Poletti et al., 2004; Sharma et al., 2017; Bouis and Saltzman, 2017).

Since agriculture is the main supplier of these micronutrients, it is essential to recognize the importance of soil and plant nutrition as influential to human health. Biofortification, through the development and production of bioavailable micronutrient enriched plants, can be a practical agronomic tool to combat nutrition problems globally (Bouis and Saltzman, 2017). In this context, the adequate crop fertilization and nutrition, mainly regarding the use of nitrogen, besides increasing the grain yield, results in the improvement of the nutritional and technological quality, inclusive on rice grain protein content (Mingotte et al., 2012, 2013, 2015). The introduction of biofortified agricultural products and improved varieties with higher mineral and vitamin content, in addition to complementing existing nutrition interventions, can provide greater sustainability and low cost for producers and consumers.

The International Center for Tropical Agriculture (CIAT) and the International Food Policy Research Institute (IFPRI) coordinate the "Harvest Plus" food biofortification program, a global alliance of research institutions and executing agencies that have teamed up to improve and disseminate products of better nutritional quality. According to Andersson et al. (2017), the HarvestPlus works in partnership with more than 200 scientific and implementation organizations around the world to improve nutrition and public health by developing and promoting biofortified food crops that are rich in vitamins and minerals, and providing global leadership on biofortification evidence and technology. The initial phase of the program includes six basic crops for human consumption: beans (*Phaseolus vulgaris*), cassava (*Manihot esculenta*), maize (*Zea mays*, L.), rice (*Oryza sativa*), sweet potatoes (*Ipomoea batatas*) and wheat (*Triticum* spp.). The objective is to generate technologies and knowledge for the development of conventional cultivars with better protein quality and higher levels of iron, zinc and provitamin A in the grains, which should be

cultivated and consumed in several developing countries.

The increase in bioavailability of essential elements in food or the implantation of species grown under selected soils with high concentration of these nutrients may be attractive tools in the production of foods based on biofortified plant, especially in the case of heavy metals. In the particular case of biofortification in Se, it is necessary to determine the specific forms in which this element is incorporated and metabolized by the plant, as this determines its bioavailability for human absorption and its biological activity in humans (Finley, 2006), for example, the production of selenogluthathione peroxidase, a potent antioxidant enzyme (Whanger, 2002).

The biofortified rice with minerals and provitamin A can complement nutrition, as well as being a sustainable and low-cost form of intervention, especially the economically disadvantaged populations. Studies suggest that doubling iron content in rice grain may increase the supply of this nutrient by 50% to the poor populations. Even with genotypic variations, in the grain rice, duplication of iron and zinc contents occurs (Gregório et al., 2000). The combination of high iron and zinc content in golden rice can help in the fight against malnutrition (Khalekuzzaman et al., 2006). The rice biofortification, with high levels of  $\beta$ -carotene, iron, zinc and lysine, combined with the development of superior agronomic and yield traits, can support the healthy eating and subsistence of millions of people who need it (Datta et al., 2006).

The first step in obtaining golden rice was to isolate the three genes responsible for producing the enzymes responsible for  $\beta$ -carotene synthesis. Two of these genes were isolated from the daffodil (*Narcissus pseudonarcissus*) and a gene from the bacterium *Erwinia uredovora*. The isolation of these genes was carried out with restriction enzymes, that is, enzymes that act on DNA fragmentation in sequences of specific nitrogenous bases. The transformation method adopted was via *Agrobacterium*, in contact with embryo cells in rice seeds. The three incorporated genes pass to code for enzymes responsible for the transformation of geranyl-geranyl diphosphate into  $\beta$ -carotene, which makes the rice grain yellow.

In this context, Burkhardt et al. (1997) verified that the Japonica rice model variety Taipei 309 was transformed by microprojectile bombardment with a cDNA coding for phytoene synthase from daffodil (*Narcissus pseudonarcissus*) under the control of either a constitutive or an endosperm-specific promoter. In transgenic rice plants, the daffodil enzyme is active, as measured by the *in vivo* accumulation of phytoene in rice endosperm. Thus, it is demonstrated for the first time, that it is in principle, possible to engineer a critical step in provitamin A biosynthesis in a non-photosynthetic, carotenoid-lacking plant tissue. These results have important implications for long-term prospects of overcoming worldwide vitamin A deficiency. The following

are some of the main techniques that can be inserted into the rice breeding programs in order to improve the nutritional quality of the food to be produced.

### RICE BREEDING FOR HIGHER Fe AND Zn CONTENT IN THE GRAIN

Approximately, 150 million hectares of rice are grown in the world, where 75% comes from the irrigated farming system. Under flooded conditions, several changes occur in soil chemical reactions, for example, the reduction of iron, which previously precipitated as hydroxides and oxides, passes to its more soluble form ( $\text{Fe}^{2+}$ ), which also increases its availability to the plants (Ponnanperuma, 1972). The concentration of soluble iron may be so high that, depending on the genotype, it can cause toxicity to the plants, leading to losses in productivity. The importance of breeding programs in the selection of rice genotypes that are tolerant to the high iron content in the soil, especially under flood conditions, is important.

In the Philippines, the breeding program of the IRRI develops studies on biofortification, producing rice-enriched seeds with iron (Fe). In the case of Fe, the bioavailability of ferritin from transgenic rice has been already tested in deficient rats, and rice diets were as effective as the  $\text{FeSO}_4$  diet in hematocrit replacement (Murray-Kolb et al., 2000). Since ferritin is used as a natural source of iron in the early development of animals and plants, it has high bioavailability.

Researchers have reported genetic variability in the rice for tolerance to excess Fe in the soil. Researchers concluded that the cultivar "CK4" rice tolerated conditions of excess Fe when cultivated under flooded conditions. This tolerance is due in part to the lower accumulation of Fe in the leaves and due to the superior photosynthetic potential in the presence of Fe in the foliar tissue.

Differences between the rice cultivars under Zn deficiency conditions, especially under high pH soil conditions, were associated with susceptibility to  $\text{HCO}_3^-$  (Forno et al., 1975). Bicarbonate concentrations of 5 to 10 mM may inhibit root growth of the inefficient rice cultivars in Zn uptake, but on the other hand, this same condition can stimulate root growth in efficient plants (Yang et al., 1994).

The higher zinc acquisition by the rice plant is related to tolerance to the high concentration of  $\text{HCO}_3^-$  in the soil (Yang et al., 1994). The differential susceptibility between beans and soybean (*Glycine max*) for zinc deficiency was associated with the restricted translocation of this nutrient from the roots. Genotypic differences for zinc use efficiency have been related to the absorption efficiency and the translocation capacity of this nutrient in the roots. Under Zn deficiency, there is an increase in the release of phytosiderophores in the form of root exudates in grass species, possibly as an adaptive response to Zn deficiency. This physiological response triggers the

mobilization of zinc and, consequently, a greater accumulation of this nutrient in the seeds.

Impa et al. (2013) studying the internal Zn allocation influences on Zn deficiency tolerance and grain Zn loading in rice (*Oryza sativa*) suggested that some Zn-efficient rice genotypes have greater ability to translocate Zn from older to actively growing tissues than genotypes sensitive to Zn deficiency. In this way, quantitative trait loci (QTLs) associated with Zn enhancement in rice has been reported, but none of them have an effect larger than 30% phenotypic variation (Stangoulis et al., 2007; Norton et al., 2014). Research efforts continue to identify major QTLs associated with grain zinc content and for better understanding of zinc uptake, transport and remobilization in the grain (Palmgren et al., 2008; Andersson et al., 2017).

In relation to the limitation of the availability of Fe in the soil, the plants evolved two strategies, known as "Strategy I" and "Strategy II", of the Fe absorption. The mechanism known as Strategy I includes extrusion of protons to solubilize  $\text{Fe}^{3+}$  in the solution of the soil,  $\text{Fe}^{3+}$  reduction solubilized by  $\text{Fe}^{3+}$  membrane-bound reductase chelate, resulting in the subsequent  $\text{Fe}^{2+}$  transport by the plant root cells by  $\text{Fe}^{2+}$  transporters.

Several studies have shown that plants belonging to Strategy I (non-grasses) when under conditions of Fe deficiency develop morphological and physiological responses. Among the observed responses is the development of root hair, through a transition or transfer cells, increasing the contact surface with the middle that surrounds the roots. This morphological modification favors the release of protons in greater quantity by the ATPases, which are "triggered" by response genes involved in the Fe transport, acidifying the middle to make the Fe more soluble. Also, expression of FRO genes involved in the Fe-reductase activity and IRT genes encoding proteins for the Fe transport occurs (García et al., 2011).

In grasses, Strategy II occurs, characterized by the formation of Fe chelates, involving the release of phytosiderophores and subsequent absorption of the  $\text{Fe}^{3+}$  through membrane sites specialized in their transport (Marschner, 1995).

Zuo and Zhang (2011) highlighted the great genetic variability in relation to Fe content in the edible portions of most cultivated species, including wheat, beans, rice, corn, cassava, yams, medicinal herbs and lentils (*Lens culinaris*). This genetic variation can boost the development of plants with high Fe content for the benefit of human health. Generally, grains of cereal such as wheat and rice have lower Fe concentrations when compared with legume seeds (Frossard et al., 2000).

The potential for increasing Fe content in rice grains is about four to five times, depending on the variability. When the difference between traditional and modern rice varieties is taken into account, the traditional ones contain higher Fe in the grains, while the modern

varieties have lower Fe concentrations (Gregorio et al., 2000, 2008). This particular observation can be attributed to the fact that direct selection for higher Fe content in grains was not part of previous rice breeding programs (Zuo and Zhang, 2011).

### **RICE BREEDING FOR HIGHER $\beta$ -CAROTENE CONTENT IN THE GRAIN**

Vitamin A is also used in genetic engineering in the production of fortified seeds. Researchers at the University of Freiburg, Germany, who studied biofortification, introduced  $\beta$ -carotene into the rice endosperm to produce the "Golden Rice". Studies are being carried out to ensure that the rice enriched with  $\beta$ -carotene is used in developing countries to combat vitamin A deficiency (Beyer et al., 2002; Datta et al., 2007). Brown rice is rich in micronutrients and carotenoids (Tan et al., 2004), but the polishing process considerably reduces its nutritional value. The control of gene expression of ferritin on the control of the glutelin promoter in rice has been efficient in increasing nutritional levels not only in whole grains of rice but also in polished grain (Goto et al., 1999; Vasconcelos et al., 2003; Khalekuzzaman et al., 2006). Similar principles have been used in the development of the golden rice (Datta et al., 2007).

Currently, none of the rice genotypes presents  $\beta$ -carotene contents in the polished grains. There is no doubt that there is potential in the use of genetic variability of the carotenoid content in rice grains (Tan et al., 2004). More than half of all women and children in Southeast Asia and Southern countries are anemic. Anemia limits growth and cognitive development in children and increases the incidence of death in severely anemic women during childbirth. However, the bioavailability of  $\beta$ -carotene should be studied with greater depth. New studies suggest that a small supplement of vitamin A or  $\beta$ -carotene may reach twice the endogenous absorption of iron from cereals (Graham and Rosser, 2000). Thus, consumption of  $\beta$ -carotene enriched rice may reduce deficiencies in vitamin A and protect against iron deficiency anemia.

### **RICE BREEDING FOR HIGHER FOLATE CONTENT IN THE GRAIN**

Folate deficiency is a health problem that affects many people in the world and is associated with deficiency in the B-complex vitamins. Food fortification by industrial supplementation and consumption of folic acid tablets are alternatives, but may not be feasible in less developed countries. Recent advances show that biofortification of agricultural products aimed at raising folate content is a viable strategy to combat folic acid deficiency around the world. The genes and enzymes involved in folate

biosynthesis are sufficiently understood. Thus, metabolic engineering mechanisms and the results of preliminary studies of genetic engineering in plants are encouraging (Bekaert et al., 2008).

The steps of folate synthesis are the same in plants and bacteria, and pathway enzymes and their genes are all known in both groups (Storozhenko et al., 2007). Essentially, the three fractions of the tetrahydrofolate (THF) molecule are pteridine, p-aminobenzoate (P-ABA) and portions of glutamate. In bacteria, the process occurs in the cytosol, but in plants, three cellular compartments are involved: the plastids, mitochondria and cytosol (Jabrin et al., 2003). The plants have developed mechanisms to deal with the instability of folic acid, and it is possible to reduce its rate of degradation.

In this aspect, the initial studies on tomato fruits and *Arabidopsis thaliana* by the expression of the enzyme cyclohidrolase I, known as GTPCH I, are promising (Diaz de La Garza et al., 2004). In both cases, with the insertion of the GenBank genes BE136861 and AE000304, the levels of pteridine in tomato (*Solanum lycopersicum*) and *Arabidopsis* (transgenic) were, respectively, 140 and 1250 times higher than the wild type, however, the increase in the content of folate was only two to four times, indicating the need for additional engineering of this metabolic pathway.

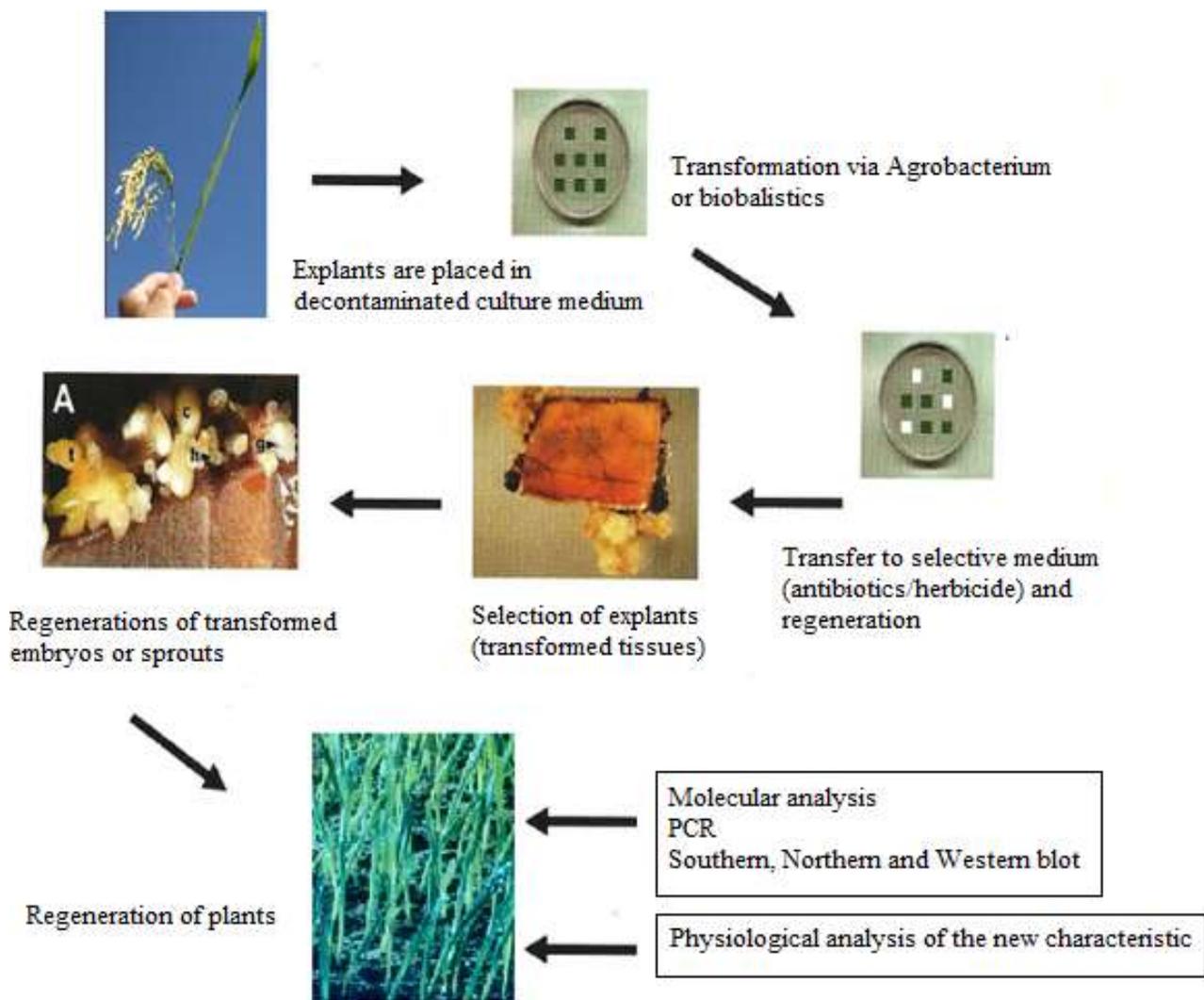
In transgenic tomato plants, p-ABA reduction occurred, and with the exogenous delivery for GTPCHI overexpression, resulted in an increase in folate content, with an additional 2.5 to 10-fold (Diaz de La Garza et al., 2004). This observation points not only to the need for simultaneous reinforcement of both folate precursors (pterine and p-ABA) but also demonstrates a great physiological potential to increase folate concentration within plant cells.

### **TECHNIQUES USED FOR PLANT TRANSFORMATION**

Genetic transformation of plants is one of the major advances in agricultural technology in recent years. The production of genetically modified plants (GMOs) has a great economic importance due to the possibility of incorporating new agronomic traits into the plants cultivated, such as resistance to herbicides, insects or phytopathogens, as well as improvement in food quality (Pereira and Vieira, 2006).

The transformation of plants combines techniques of tissue culture, methods of transformation and selection *in vitro* (Figure 1). The transformation can be done directly by the physical introduction of DNA into plant cells, or by biological methods, where microorganisms such as viruses or bacteria are used as transformation vehicles. Three transformation systems are more widely known and therefore common, these are, transformation via protoplasts, bioballistics and via *Agrobacterium tumefaciens*.

Nowadays different characteristics of socio-economic



**Figure 1.** Transformation of plants involved in tissue culture techniques and *in vitro* transformation methods. Source: Adapted from Pereira and Vieira (2006).

interest have already been introduced in different plant species by genetic transformation. These characteristics mainly aimed at improving performance in the field of cultivated plants, by means of resistance to biotic and abiotic stresses. Characteristics related to the development of the plant and the quality of the product can also be modified in transgenic plants. The trend is to increase the number of traits that can be manipulated through genetic engineering, increasing the range of products to be made available to the farmer and the consumer.

### Transformation via protoplasts

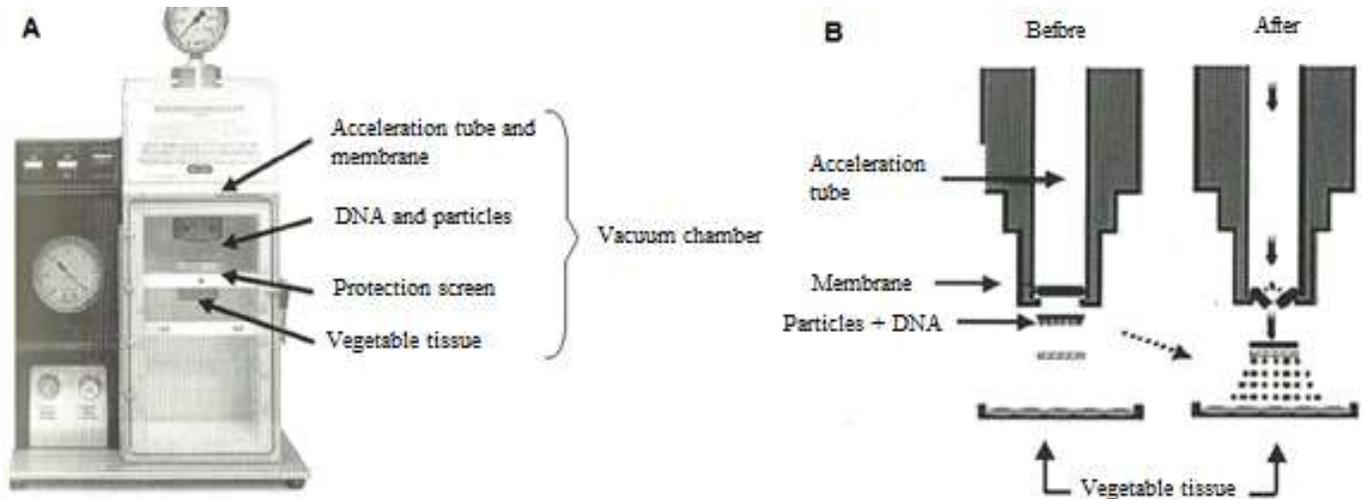
Protoplasts, plant cells without a cell wall, can be considered as the ideal type of plant cell for transformation. The absence of the cell wall allows free transit to a DNA sequence that can penetrate most cells,

including the competent cells for transformation and regeneration (Potrykus, 1991). This method of transformation can be carried out chemically, using polyethylene glycol or polyvinyl alcohol, or by electroporation, which consists of producing pores in the membrane through fast high voltage electrical impulses.

As a limitation of the transformation technique via protoplasts, it is worth mentioning, the difficulty in regeneration, because few plant species have a high regenerative rate. The major success of protoplast transformation was observed in rice, where transgenic plants with hygromycin phosphotransferase and b-glucuronidase (GUS) genes were obtained for both *indica* and *japonica* subspecies (Zhang and Wu, 1988).

### Transformation via biobalistics

The insensitivity of certain plant species, especially



**Figure 2.** Particle acceleration equipment with main components (a) and cross section showing particle acceleration system (b). Source: Adapted from Pereira and Vieira (2006).

monocotyledons and angiosperms, to *Agrobacterium* infection limits the use of this bacterium as a vector of genetic transformation. Thus, direct transformation systems, based on chemical or physical methods, were developed in parallel with the *Agrobacterium* system.

The bioballistics (or biolistic) method, or particle bombardment, has been highlighted as one of the most important methods of transformation of plants because, in addition to transforming plant tissues, it also allows the transformation of microorganisms or animal tissues (Klein et al., 1992). It is a technique with potential for use in any plant species, provided there is an *in vitro* regeneration system from the target tissue. Many devices have been proposed for particle acceleration. Nowadays, the most used models are the acceleration of particles by electric discharge or air pressure, using membranes of different thickness (Figure 2).

In comparison of this method with the *Agrobacterium* transformation system, bioballistics does not depend so much on the genotypes and their interaction with the bacteria, but rather, there is a good *in vitro* regeneration system of the material to be transformed (Pereira and Vieira, 2006).

### Transformation via *A. tumefaciens*

The transformation methods mostly used in the production of GMO plants are mediated by the *A. tumefaciens* and via bioballistics. The *Agrobacterium* is a typical soil bacterium that has the ability to transfer DNA fragments into plants, and it has been used as a plant transformation vector.

Obtaining a transgenic plant involves the transfer and integration of the transfer DNA (T-DNA) into the plant cell and the ability of these transformed cells to differentiate

into a plant (Figure 3).

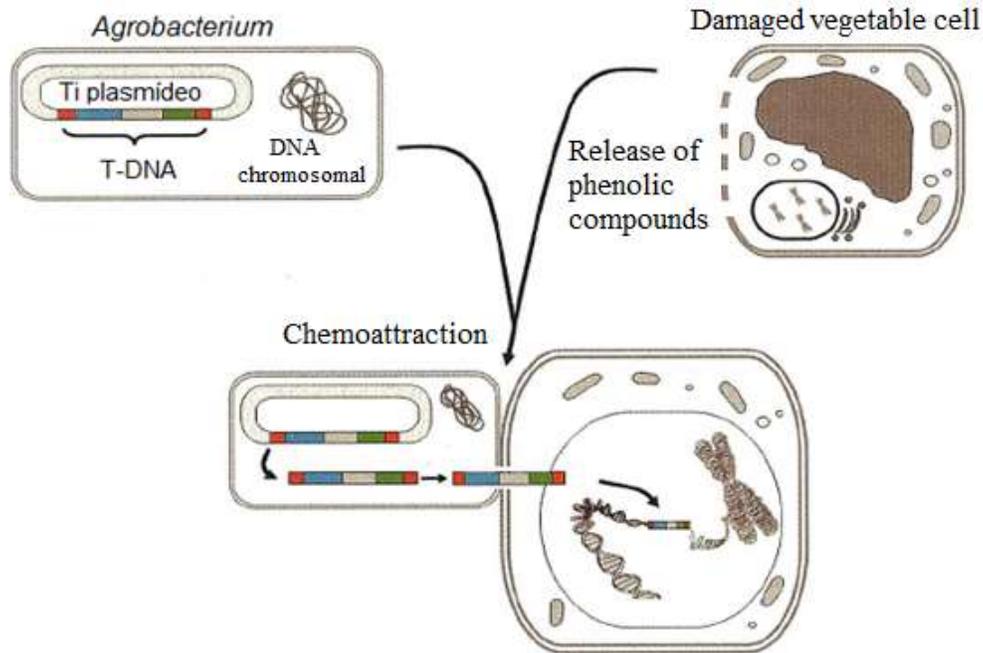
The ability of differentiation, called totipotency, allows the regeneration of plants by *in vitro* tissue culture techniques. The advancement in molecular biology knowledge is fundamental both to elucidate the in depth molecular bases of the *Agrobacterium*-host interaction process and to build transformation vectors based on the tumor-inducing (Ti) plasmid. Thus, molecular biology techniques in association with *in vitro* plant tissue culture techniques form the basis for obtaining a transgenic plant.

### Identification and *in vitro* selection of transformed tissues

In studies on genetic transformation of plants, the use of reporter genes or marker genes is necessary, besides the genes of agronomic or scientific interest. Reporter genes serve to verify that the transformation process is being done correctly by identifying transformed cells or tissues. These genes allow an analysis of transient expression of the gene temporarily, even when the gene is not integrated into the genome of the cells.

The marker genes are used to enable discrimination between transformed and untransformed cells, and consequently the selection of the former. Such genes are introduced to facilitate the work of identifying them because they are a minority in relation to the total cells undergoing a transformation. Antibiotic resistance genes are generally used. Thus, at the time of plant regeneration from a cell, the addition of antibiotic to the middle will only allow the growth of the transformed cells expressing the right protein.

One of the most commonly used reporter genes is *uidA* which encodes the  $\beta$ -glucuronidase enzyme (GUS) and



**Figure 3.** Transformation of plants via *A. tumefaciens*. Source: Adapted from Pereira and Vieira (2006).

provides a histochemical or fluorescent analysis of the transformed material. Other widely used reporter genes are green fluorescent-protein genes (*gfp*), extracted from Jellyfish *Aequorea victoria*; and the Luciferase gene (*luc*), isolated from *Photinus pyralis* (firefly). These reporter genes (*luc* and *gfp*) have been most widely used in the identification of transformed tissues.

With the development of more sensitive photodetection and imaging equipment, *luc* genes are being used for *in vivo* labeling and visualization in real time of cells and tissues; for marking and dissemination studies of pathogenic microorganisms in plants and animals; as sensitive markers of cancer cells, assisting in the study of metastatization and the development of new therapies; and as environmental biosensors for the detection of heavy metals such as mercury and arsenic or pesticides in contaminated waters (Viviani, 2008).

One of the most recent applications in the field of proteomics involves the use of *luc* and *gfp*, because the methods that involve reporter genes allow the identification of the site (root, leaf or fruits) of occurrence of gene expression, being related to gene regulation. Methods related to the expression allow evaluating the occurrence of gene expression in certain situations, such as the presence or absence of a certain nutrient, which is possible for selecting genotypes.

## CONCEPTS OF GENE EXPRESSION AND REGULATION

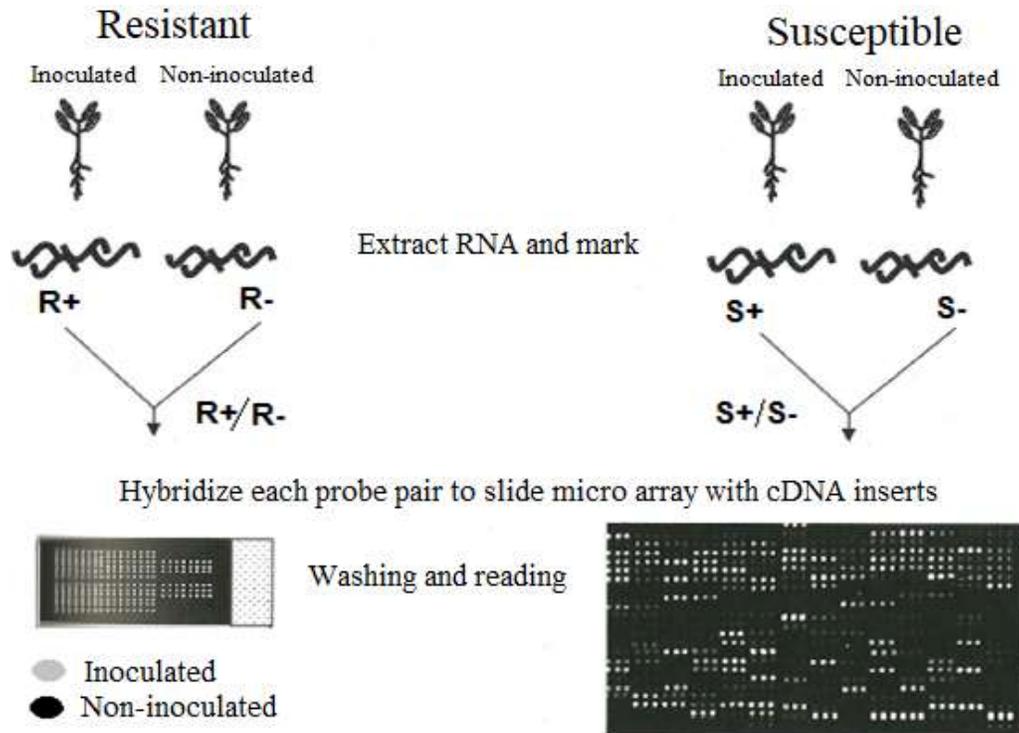
The analysis of gene expression is importance in many

fields of biological research. Knowledge on gene expression patterns should provide a better understanding of complex regulatory systems and probably lead to the identification of relevant genes for plant breeding. The discovery of the polymerase chain reaction (PCR) has brought enormous benefits and scientific development, such as genome sequencing and gene expression. This technique allows amplifying a specific segment of a DNA molecule using two primers (short nucleotide sequences) complementary to the ends of the segment to be amplified and a DNA polymerase.

The technique called RT-PCR allows amplifying the coding passages directly from mRNA molecules. After extraction of the RNA, a single strand of DNA complementary to the mRNA (cDNA) is synthesized using the reverse transcriptase enzyme of viral origin. The RNA tape from The RNA / DNA hybrid is digested with RNase, and the cDNA strand is replicated by a DNA polymerase. When compared with the other two techniques commonly used to quantify mRNA, Northern blot analysis and RNase protection assay, RT-PCR can be used for the quantification of mRNA in much smaller samples. In fact, this technique is sensitive enough to allow approximate quantification of RNA from a single cell (Weis et al., 1992).

The technological innovation resulting from PCR, called real-time PCR, has been gaining ground in clinical diagnostics and research laboratories because it has the ability to obtain results more accurately and rapidly in relation to PCR.

Several methodologies have been developed for the



**Figure 4.** Microarray experiment scheme for analysis of genes involved in the response of plants to situations with and without nutrients, or absence and presence of pathogens. Source: Adapted from Yang et al. (2002).

large-scale identification of differentially expressed genes. Among the most used, the microarray technique, which is based on the quantification of mRNA produced by the cell (gene expression products), provides a powerful platform for analyzing the expression pattern of thousands of genes simultaneously.

In the microarray technique, the mRNA is extracted from the tissues to be studied comparatively. The mRNAs are transformed into cDNA. Each cDNA pool is marked with clusters of fluorescent nature. After labeling, the samples in the microarrays are denatured by heat. The cDNAs studied are grouped and placed in contact with the microarrays by submerging the chip in a hybridization solution. Both cDNAs of the tissues studied and the "pores" of the slide are in plain ribbon form. If there is complementarity between the sequences, the labeled cDNA strands will hybridize to the cDNAs of a "pore" of the slide. After washing and drying the chip, it is taken to a dark room and irradiated with a laser. The fluorescent markers will absorb the radiation. Each marker emits radiation at a different wavelength, allowing the quantification of hybridized cDNA in any "pore" of the slide. As gene fixed in each of the "pores" is known, it is possible to know the expression of a control tissue in relation to the variant. Colors are assigned computationally to the emission ranges of each of the markers and the variations between the colors determine the levels of expression (Figure 4) (Yang et al., 2002).

The microarray technique allows the analysis, in parallel, of thousands of genes in two populations of labeled RNAs, while RT-PCR provides the simultaneous measurement of gene expression in different samples for a limited number of genes and it is especially desirable when only a small number of cells are available. Both techniques have the advantage of speed and the high degree of potential automation when compared with traditional quantification methods such as Northern blot analysis, *in situ* hybridization and ribonuclease protection assay.

*In situ* hybridization is a method of locating and detecting specific nucleic acid sequences (DNA and RNA) of tissue sections preserved or prepared from cells by hybridizing a complementary band of a specific nucleotide probe (DNA or RNA) of the sequence of interest. This technique, while being a complex method, allows a localization of specific cell transcripts within a tissue. The basic principles for *in situ* hybridization are the same for DNA or RNA, varying in type of cells and tissues to be studied, which require probes to detect specific nucleotide sequences within cells and tissues (Parker and Barnes, 1999).

### Protein expression

The identification of specific proteins has been one of the

main objectives of scientific and diagnostic practices. Blotting techniques are used to identify unique proteins or sequences of nucleic acids. The three main blotting techniques that have been developed are commonly called Southern, Northern and Western Blotting, and they enable the identification of DNA, RNA, and proteins, respectively.

Southern blotting (Southern, 1975) allows DNA fragments to be identified with DNA probes, which are hybridize by hydrogen bonding to form complementary fragments of chromosomal DNA. Prior to Southern blotting analysis, DNA fragments must be produced from chromosomes by enzymatic digestion with restriction endonucleases. These enzymes are obtained from microorganisms and digest the double-stranded DNA at specific sites determined by the nucleic acid sequence.

Northern blotting analysis employs essentially the same procedure described for Southern blotting, except that complementary DNA is used for RNA probe. Northern blotting technique (Thomas, 1983) allows individual messenger RNA molecules (mRNA) be identified and measured after hybridization of their corresponding DNA sequences. The messenger RNA is initially separated by electrophoresis based on its size and they are transferred to paper before the probes genes that are used to locate the RNA of interest.

Western blotting allows specific proteins to be identified with specific antibodies used as analytical probes. The Western blotting analysis requires specific antibodies for the protein under analysis.

## Proteomics

Although, the identification of all proteins encoded in the genome of an organism seems a rather difficult task to perform, even in simpler organisms, the information obtained through proteomic studies is increasingly complete (Suresh et al., 2005). This new knowledge is related to cell signaling pathways, regulatory protein assemblies, post-translational modifications, as well as other crucial information on the physiological and pathophysiological states of cells and organisms.

The term proteome was proposed in 1995 by Wilkins as being the entire content of proteins expressed by a genome. After the euphoria caused by the sequencing of the genomes of several organisms, the scientific community realized that in order to understand the gene function in its fullness, the large-scale study of the expressed proteins was necessary. Among the main techniques used in proteomics are two-dimensional electrophoresis and mass spectrometry.

Electrophoresis is a separation technique based on the migration of charged molecules into a solution, depending on the application of an electric field. Protein electrophoresis was first performed in 1937 by Arne Tiselius, who devised a method called free electro-

phoresis, which consisted of the decomposition of blood serum into five main protein fractions; this study was important for him to win a Nobel Prize. In the last decades, this technique experienced constant improvements, enabling more precise analyzes, as in the case of denaturing electrophoresis in polyacrylamide gel and two-dimensional electrophoresis.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) is a widely used method for the analysis of molecular masses of oligomeric proteins. The gel is a matrix consisting of a crosslinked acrylamide polymer of N, N-methyl-bis-acrylamide whose mesh porosity can be chosen. The higher the acrylamide concentration, the smaller the pores of the formed mesh. After the treatment with amphipathic detergent, proteins are applied to the top of a polyacrylamide gel and subjected to an electric current, making them to migrate through the acrylamide mesh towards the direction of the positive pole. Depending on its size, each protein will move differently, with smaller proteins migrating faster, while larger ones will have more difficulty in moving the gel mesh and thus they will move more slowly. When electrophoretic mobility is plotted against the logarithm of known molecular weights of various polypeptide chains (labeling proteins), a straight line is obtained which can be used as the standard for calculating molecular weight of the subunits of the protein of interest.

## Mutant study

Genetic variability forms the base in the essence of evolutionary processes and plant breeding; it is essential for the natural and/or artificial selection to be effective (Jennings et al., 1981). In addition to the pre-existing genetic variability in the germplasm, it is possible to add variability through artificial mutations, gene recombination, genetic transformation and soma-clonal mutations. For more than 50 years, different strategies have been used in this regard, and the many rice cultivars have been produced from induced mutations and/or selection of genetic constituents from mutant populations. In many cases, a point mutation can correct or improve some traits, allowing the selection of superior genetic constitutions in the early generations. This strategy was used to change the frequency of some genes that are negligible by the expression of traits in the IAC-24 wheat cultivar, allowing the selection of at least eight lines that maintained the original characteristics and had traits such as the height of plants and tolerance to pathogens (Tulmann-Neto et al., 1995). In triticale, mutation induction was efficient in reducing the height of plants like those produced by artificial crosses (Pandini et al., 1997).

Researchers in Brazil have used  $^{60}\text{Co}$  mutation induction to generate genetic variability in the BRS 7 "Taim" rice cultivar, belonging to the modern group

(Filipino), characterized by short and erect leaves with average height of about 80 cm, with high tillering capacity and a biological cycle, in the southern part of the state of Rio Grande do Sul; with 125-130 days of emergence, complete maturation and sensitivity to cold, mainly in the reproductive phase. In the M2 generation, 623 mutants were selected for different traits, including the height of plant and cycle (Zimmer et al., 2003).

In a study developed by Martins et al. (2005) in which the variability for morphological traits in rice mutants was studied, it was observed that the seed irradiation of the cultivar BRS 7 "Taim" with  $^{60}\text{Co}$  was efficient in the generation of mutants for the traits: height of plant, cycle, number of tiller, number of panicles and index of fertile tiller.

### Patch-clamp

Some techniques of the study on transporters and other membrane proteins related to the transport of nutrients in plants can be used in parallel with genetic breeding in order to obtain plants with higher nutritional value.

The patch-clamp method consists of, a tiny area of the cell membrane (patch) and making recordings of chains that flow through it (Barry and Lynch, 1991). A glass micropipette, filled with a suitable electrolytic solution and with resistance ranging from 4 to 6 M $\Omega$ , is pressed against the membrane and melts with it, forming an extremely high resistance and mechanical stability seal. Subsequently, the membrane patch is ruptured by suction with the pipette still sealed to the cell, providing access to the interior of it. A high strength seal is required for two reasons: first, the higher the seal strength, the more complete is the membrane patch insulation with respect to the external solution, and second, a high resistance decreases the chain that can pass between the pipette and the membrane. In this way, all the ions that flow, when the ion channels open, must flow into the pipette. The resulting electric chain, although small, can be measured with an ultra-sensitive amplifier connected to the pipette.

Fuchs et al. (2005) confirmed that rice K<sup>+</sup> uptake channel OsAKT1 is sensitive to salt stress by the patch-clamp method. They used this technique on rice root protoplasts to identify a K<sup>+</sup> inward rectifier with similar channel properties as heterologously expressed OsAKT1. This technique was developed for studies on the behavior of ion channels and presents a great potential to be used in plant breeding research regarding the selection of individuals with a higher number of specific carriers for certain nutrients in root canal tissues. Also, patch-clamp technique can help in the discovery of potential membrane transporters to be inserted in cultivated species, thus improving, the efficiency of the absorption of nutrients even under conditions of less availability in the soil.

### FINAL CONSIDERATIONS

Some researchers pointed out the possibility of performing agronomic biofortification, through fertilization with positive effects on the nutritional quality of the food. However, the genetic biofortification together with the agronomic one extends the possibilities of development of new cultivars more efficient in accumulating minerals and vitamins in the edible part.

It is necessary to verify the occurrence of variability among the rice genotypes for the Fe, Zn and the B complex vitamins, so that it will be possible to select these materials within breeding programs. The simple selection of these superior genotypes in relation to nutritional quality, even though by means of conventional breeding techniques can have benefits on rice consumption by the human population.

With the advent of genetic engineering, following the methods presented so far, it is possible to generate a rice plant that produces high-concentration  $\beta$ -carotene grains. The knowledge of the enzymes involved in the  $\beta$ -carotene biosynthesis was substantial to the success of obtaining this product that can combat problems of nutritional deficiency in low-income populations.

As strategies to be adopted in rice breeding, aiming at biofortification of the grain is important in the techniques of genetic engineering and biotechnology. In parallel with the knowledge on genetics, some plant nutrition concepts should be adopted by breeding programs, especially the biomolecular aspects of plant nutrition.

### CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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## Full Length Research Paper

# Preventive effect of black rice antioxidant extract on oxidative stress induced by ethyl alcohol

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The present study investigated the black rice ethanolic extract (BREE) protective effect as natural antioxidants against oxidative stress induced by ethyl alcoholic (EA) in male rats. BREE flavonoids and phenols were determined by high performance liquid chromatography (HPLC). The study was conducted on 40 male Wistar rats weighing  $170 \pm 2$  g, the animals divided into 4 equal groups. The first group was given distilled water (DW) and used as a negative control (NC) group. The second group was administrated EA (6 g/kg bw/day) and used as positive control (PC) group. The other groups of rats were administrated [BREE 125 or 250 mg/kg bw + EA 6 g/kg bw/day]. Blood samples were collected after 10 days. Lipid profile, thiobarbituric acid (TBA), superoxide dismutase (SOD), glutathione (GSH) and F<sub>2</sub>-isoprostanes (F<sub>2</sub>-isoPs) were determined. The results indicate that the rats treated with EA 6 g/kg bw/day showed a significant ( $p \leq 0.05$ ) increase in the levels of total cholesterol (TC), low density lipoprotein-cholesterol (LDL-C), TBA and F<sub>2</sub>-isoprostanes and significant ( $p \leq 0.05$ ) decrease in high-density lipoprotein-cholesterol (HDL-C), SOD and GSH levels. The rise in serum TC, LDL-C, TBA and F<sub>2</sub>-isoprostanes were significantly attenuated by BREE 125 or 250 mg/kg bw + EA 6 g/kg bw/day. Also, the levels of serum HDL-C, SOD and GSH in BREE 125 or 250 mg/kg bw + EA 6 g/kg bw/day groups showed a significant ( $p \leq 0.05$ ) increase as compared to PC group. The current results ascertained the beneficial effects of BREE in controlling oxidative stress induced by ethyl alcohol in male rats.

**Key words:** Antioxidants, black rice extract, ethanol, oxidative stress, free radicals, F<sub>2</sub>-isoprostanes, rats.

## INTRODUCTION

Rice (*Oryza sativa* Linn.) is the principle and important cereal crop for over half of the world (Clampett et al., 2002; Hansakul et al., 2011) (Figure 1). It plays an important role in the relation between the diet and health,

because it contains many phytochemicals as bio-active compounds such as  $\gamma$ -oryzanol, phenylpropanoids, tocopherols, tocotrienols, phenolics, and flavonoids compounds (Clampett et al., 2002). Colored rice having

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red, purple and black rice hulled are considered to be a healthy food, especially black rice which has many health benefits because it contains a lot of antioxidative phytochemicals (higher amount of phenolic compounds and flavonoids) and anthocyanins (Ryu et al., 2003; Lum and Chong, 2012) which adds as reducing agents, metal ion chelators and free radical scavengers (Lum and Chong, 2012). Additionally, anthocyanins may reduce the risks of hypercholesterolemia [decreased total cholesterol (TC), low density lipoprotein (LDL) and triglycerides (TG)], antioxidants, cancer, inflammatory process, atherosclerosis and anti-inflammatory activities (Chen et al., 2006; Walter and Marchesan, 2011).

Moreover, phenolic compounds, flavonoids and anthocyanins have many properties such as antioxidant activity, nonmutagenic and nontoxicity, therefore, considered to be significant health implications (De Pascual-Teresa et al., 2002) and widely used in food industry (Kong et al., 2003). Previous studies reported that black rice pigments improved lipid profile and reduced oxidative stress in the liver induced by alcohol, which is due to imbalance between antioxidants and pro-oxidant systems in liver (Valcheva-Kuzmanova et al., 2004; Nordmann et al., 1992), control of lipid in blood and related diseases (Ling et al., 2001) and prevention of Alzheimer's (Miyake et al., 2012).

Ethanol alcohol has an important role of lipid peroxidation and oxidative stress because it is soluble in water and lipids. Thus, it can be spread through stomach and oesophagous mucous membrane and appears in urine and expired air. Then, it is ingested and oxidized in liver (Abraham et al., 2002). Oxidative stress is induced by alcohol due to metabolism of ethanol. Many studies indicated that there are three pathways of ethanol metabolic in the body of human, which include the following enzymes: (1) alcohol dehydrogenase, which catalyzes ethanol to form acetaldehyde which results in the free radical formation. Hydride ion of ethanol transferred to  $\text{NAD}^+$  (Cunningham et al., 2001), also causes concomitant changes in the levels of  $\text{NADH}/\text{NAD}^+$  redox ratios and  $\text{NADH}$  (Das et al., 2005; Mantle and Preedy, 1999); (2) Microsomal ethanol oxidation system (MEOS), cytochrome p450 isoenzymes ( $2\text{E}_1$ ,  $1\text{A}_2$ ,  $3\text{A}_4$ ) is catalyzed by ethanol oxidation (Lieber and DeCarli, 1970).  $2\text{E}_1$  isoenzyme may be a significant catalyst for reactive oxygen species (ROS) formation in the consumer of alcohol and generated higher amounts of  $\text{H}_2\text{O}_2$  (Nordsblom and Coon, 1977) and increased hydroxyl radicals generation (Klein et al., 1983). The formation of ROS such as  $\text{H}_2\text{O}_2$  and superoxide anion ( $\text{O}_2^-$ ) represents important cause of oxidative injury in many diseases associated with free radical formation. In the presence of trace amounts of transition metals (most frequently Fe)  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  generate highly-reactive hydroxyl radicals, which are then responsible for the oxidation of biological constituents (Albano, 2006), and catalase (Das et al., 2005; Mantle and Preedy, 1999).

Ethanol ingestion leads to increment of TG in blood (Zima, 1993), serum cholesterol and hepatic cholesterol ester levels (Schroeder et al., 1995) and accumulation of very low density lipoprotein (vLDL) and LDL in blood (Frohlich, 1996). Catabrese et al. (1995) indicated high levels of fatty acid ethyl esters and free fatty acids (FFA) in liver, brain, heart and kidney of rats treated with ethanol. The measurement of changes in endogenous antioxidant enzyme activity is considered a fairly sensitive biomarker of the response to oxidative stress. The cells were protected from ROS damage by glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) are primary antioxidant enzymes (Das and Vasudevan, 2007).

Zhaohua et al. (2010) evaluated the protective effect of anthocyanin-rich extract from black rice (AEBR) on chronic ethanol-induced biochemical changes in male Wistar rats and reported that rats treated with AEBR showed a better profile of the antioxidant system with normal glutathione S-transferase (GST), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activities. The results demonstrate that AEBR has a beneficial effect in reducing the adverse effect of alcohol. Quantification of F2-isoprostanes (F2-isoP), prostanoids produced by non-enzymatic free radical-catalyzed peroxidation of arachidonic acid, in plasma or urine is sensitive and specific index of lipid peroxidation (LP) *in vivo* (Roberts and Morrow, 1993; Delanty et al., 1996). Moreover, ethanol-stimulated lipid peroxidation is linked to the impairment of mitochondrial oxidative phosphorylations (Bailey and Cunningham, 2002). Because alcohol-induced several organs injury, which has been linked to oxidative stress, the effect of black rice extract as natural antioxidant against oxidative stress induced by ethyl alcohol in male rats was investigated.

## MATERIALS AND METHODS

### Preparation of black rice ethanolic extract

Black rice grains (*O. sativa* Linn. 2015) (Figure 1) were obtained from Field Crops, Research Institute, Agriculture Research Center, Giza, Egypt. Blender (Waring 2-L Laboratory Blender- The Lab Depot Inc) was used to ground black rice (Figure 2A). The black rice extract was extracted from weighed portion (100 g) of black rice with ethyl alcohol (150 ml) at room temperature for 24 h. Filter paper Whatman No.4 was used to filter the ethanolic extract of black rice. Solvent extraction was repeated twice and the extract solution (Figure 2B) was combined and kept at 4°C until used.

### High-performance liquid chromatography (HPLC)

Flavonoids/compounds and phenols/compounds were determined by Agilent 1100 HPLC equipped at 330 and 280 nm, respectively, with multiwavelength detector, Auto-sampler, degasser, quaternary pump and column compartment set at 35°C. This compound was fractionated by column zorbox ODS 5  $\mu\text{m}$ , 4.6  $\times$  250 mm. The mobile phase flow rate was set at 1 ml/min (Pascale et al., 1999; Pirjo et al., 2002).



**Figure 1.** Black rice grains.



**A**



**B**

**Figure 2. (A)** Black rice powder. **(B)** Black rice ethanolic extract.

### Biological methods

The adult albino male rats [(40 animals), weighing  $170 \pm 2$  g] were obtained from Vaccination Center, Helwan, Giza, Egypt, then transported to Animal House of Ophthalmology Research Institute, Giza, Egypt. Rats were housed in individual cages with screen bottoms and fed for ten days on basal diet (cellulose 5%, corn starch 70%, corn seed oil 10%, casein 10%, vitamins mixture 1%, and salt mixture 4%). The rats were weighed after equilibration, then divided into four groups (ten animals per each); everyone was assigned to one of the four diet groups; first group: normal or negative Control (NC) received normal diet, Second group: Positive Control (PC) treated orally with EA (6 g/kg bw/day), third and fourth groups: treated orally with EA (6 g/kg bw/day) + BREE (125 and

250 mg/kg bw/day, respectively). Approval was obtained from Institutional Review Board (IRB): 2015-10-239. Blood samples were collected according to Schermer (1967). To obtain blood serum, each sample was centrifuged ( $1500 \times g$ ) at  $4^\circ\text{C}$  for 30 min in the dry clean centrifuge tube.

### Biochemical assays in serum

The methods described by Allain et al. (1974), Fossati and Prencipe (1982), and Lopez-Virella et al. (1977) were used to determine Total cholesterol (TC), Triglyceride (TG) and high density lipoprotein cholesterol (HDL-C), respectively. The formula of Friedewald et al. (1972) was used to calculate the low-density

**Table 1.** Phenols and flavonoids components in BREE analyzed using HPLC.

Phenols		Flavonoids	
Compound	mg/100 g	Compound	mg/100 g
Benzoic	2.798	Rosmarinic	0.129
Cinnamic	1.983	Hispertin	2.309
Pyrogallal	4.003	Kampferol	0.351
Protocatchuic	4.005	Quercetin	6.819
Hydroxytyrozol	2.623	Luteolin	0.224
Gallic	0.600	Apigenin	0.502
Coumarin	3.982	Naringin	1.901
Salicylic acid	7.813	Narenginin	1.400
Ferulic	0.015	Quercetrin	3.602
Vanilic	1.202	Hypersoid	0.371
Caffeic	0.812	Rutin	6.586
Catechin	4.589	Hisperdin	2.345
Catechol	0.609	---	---
Ellagic	2.207	---	---
Cholorogenic	4.998	---	---
Caffein	1.209	---	---
Total	43.448 (mg/100 g)	Total	26.539 (mg/100 g)

BREE, Black rice ethanolic extract.

lipoprotein cholesterol (LDL-C) levels for serum samples. Also, the methods described by Ohkuma et al. (1982) and Pegg (2005) were used to measure the enzymatic activity of superoxide dismutase (SOD) and thiobarbituric acid (TBA) in the serum blood samples. The instructions of Morrow and Roberts (1997) were used to measure F2-isoprostanes (F2-IsoPs) by using a competitive enzyme-linked immunoassay (ELISA) kit (Cayman Chemical, Ann Arbor, MI). All other chemicals used were purchased from Algomhorya company, Giza, Egypt.

### Statistical analysis

The standard error of mean (SEM) was used to express the results. One way analysis of variance (ANOVA) followed by Fischer's LSD test was used to measure the intergroup variation. Statistical significance was considered at  $P \leq 0.05$ . The statistical analysis was done using the Jandel Sigma Stat Statistical Software version 2.0.

## RESULTS AND DISCUSSION

Table 1 shows phenolic contents (PCs) and flavonoids contents (FCs) in BREE analyzed by using HPLC. PCs and FCs were 43.448 and 26.539 mg/100 g, respectively. The highest compounds of PCs and FCs are salicylic acid (7.813 mg/100 g) and quercetin (6.819 mg/100 g), respectively. PCs was also observed by Lum and Chong (2012), they found that PCs ranged from 22.59 to 329.53 mg/kg in Malaysia rice. Also, Salgado et al. (2010) found that PCs contents in black rice amount to 23.78 mg/g. China black rice contained the highest PCs as compared to white and red rice; also, ethanol (70%) extracts contained more PCs and FCs as compared to water (25

and 50°C) extracts (Chanida et al., 2013). Also, black rice had the highest FCs when compared with white and red rice; it has content FCs range from 16.98 to 158.47 mg/kg (Chanida et al., 2013; Melissa and Enio, 2011). Wang et al. (2014) determined the phenolic constituents in black rice by HPLC-MS/MS and they found that the negatives mode detected many components including p-coumaric acid, vanillic acid, gallic acid, ferulic acid, syringic acid, caffeic acid, protocatechuic acid, p-hydroxybenzoic acid, rutin, and quercetin. Also, Loypimai et al. (2016) studied the black colorant powder (BCP) and they showed higher concentrations of gallic acid (253.29 to 257.57 mg/g), caffeic acid (129.34 to 136.12 mg/g), ferulic acid (630.74 to 663.34 mg/g), total phenolics (187.18 to 201.61 mg gallic acid equivalent (GAE/g) and lutein (70.6 to 72.4 mg/g). Total anthocyanins of black rice extract contained  $416.92 \pm 0.63$  mg (Hao et al., 2015). Additionally, cyanidin-3-glucoside content was 11 times higher than peonidin-3-glucoside, which was consistent with previous reports (Hou et al., 2013; Lee, 2010).

The data in Table 2 shows the results of initial, final, gain and daily gain body weight in NC, PC and EA (6 g/kg bw) + BREE, (125 and 250 mg/kg bw) administration rats. The body weights in ethanol treated group rats were found to be significantly ( $P \leq 0.05$ ) reduced as compared to the control, which is similar to the previous reports (Lieber, 1994; Hou et al., 2010); the reason may be due to malabsorption and food intake loss (Lieber, 1994). The excessive alcohol ingestion disturbs the metabolism of most nutrients in diet resulting in malnutrition (Lieber,

**Table 2.** Effect of BREE on body weight of control and ethanolic administrated rats

Treatment	Initial body weight	Final body weight	Gain body weight	Daily Gain body weight
NC	170.40 <sup>a</sup> ±0.75	191.00 <sup>a</sup> ±0.71	20.60 <sup>a</sup> ±0.24	2.06 <sup>a</sup> ±0.02
PC	170.60 <sup>a</sup> ±0.81	143.20 <sup>c</sup> ±0.80	-27.40 <sup>d</sup> ±0.81	-2.74 <sup>d</sup> ±0.08
G1 (125 mg/kg bw BREE)	171.40 <sup>a</sup> ±0.60	183.60 <sup>b</sup> ±0.60	12.20 <sup>c</sup> ±0.37	1.22 <sup>c</sup> ±0.04
G2 (250 mg/kg bw BREE)	170.80 <sup>a</sup> ±0.73	189.00 <sup>a</sup> ±0.71	18.20 <sup>b</sup> ±0.49	1.82 <sup>b</sup> ±0.05
LSD	2.183	2.120	1.572	0.157

BREE, Black rice ethanolic extract; TC, Total cholesterol; HDL-C, high density lipoprotein; LDL-C, low density lipoprotein; Gand G2 Groups, 1 and 2. Values are mean (dev for 10 rats in each group. NC, Compared with negative control group:  $P \leq 0.05$ ; PC, Compared with positive control group:  $P \leq 0.05$  (One-way ANOVA followed by Fischer's LSD test).

**Table 3.** Effect of BREE on TC, HDL-C and LDL-C activities in control and ethanolic administrated rats.

Treatment	TC (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)
NC	148.85 <sup>d</sup> ±0.09	107.98 <sup>b</sup> ±0.37	38.91 <sup>d</sup> ±0.13
PC	288.39 <sup>a</sup> ±0.41	97.97 <sup>d</sup> ±0.25	193.78 <sup>a</sup> ±0.27
G1 (125 mg/kg bw BREE)	198.80 <sup>b</sup> ±0.07	101.38 <sup>c</sup> ±0.17	97.99 <sup>b</sup> ±0.03
G2 (250 mg/kg bw BREE)	150.16 <sup>c</sup> ±0.13	108.81 <sup>a</sup> ±0.17	41.64 <sup>c</sup> ±0.18
LSD	0.670	0.760	0.524

BREE, Black rice ethanolic extract; TC, Total cholesterol; HDL-C, high density lipoprotein; LDL-C, low density lipoprotein; Gand G2 Groups, 1 and 2. Values are mean (dev for 10 rats in each group. NC, Compared with negative control group:  $P \leq 0.05$ ; PC, Compared with positive control group:  $P \leq 0.05$  (One-way ANOVA followed by Fischer's LSD test).

2000). Also, the results indicated that rats administered EA (6 g/kg bw) + BREE, (125 and 250 mg/kg bw) gave the best results compared to NC (Table 2). This protective effect may be due to the presence of polyphenols and nutrients present in BREE (Hou et al., 2010; Jang et al., 2012; Hou et al., 2013) which might have detoxified the liver and improved the body weight to near normal levels.

#### Effect of BREE on TC, HDL-C and LDL-C levels in serum

Levels of TC, HDL-C and LDL-C in serum of experimental and NC rats are presented in Table 3. Treatment with ethyl alcohol caused a severe significant increase in TC and LDL-C levels ( $P \leq 0.05$ ) in blood serum, while decrease of blood serum HDL-C level ( $P \leq 0.05$ ) than NC. The altered redox state secondary to the oxidation of ethanol promotes lipogenesis through enhanced formation of acylglycerols. The depressed oxidative capacity of the mitochondria injured by chronic alcohol feeding also contributes to the development of the fatty liver. Accumulation of fat acts as a stimulus for the secretion of lipoproteins and the development of hyperlipemia. At the early stage of alcohol abuse, when liver damage is still minimal, hyperlipemia will prevail, whereas hypolipemia occurs in the later stages with severe liver injury (Lieber and Savolainen, 1984). On the other hand, pretreatment of rats with BREE (125 and 250 mg/kg body weight) reduced the formation of HDL-C,

LDL-C and TC of serum when compared with the ethanol group, the serum TC and LDL-C levels decreased significantly, while the serum HDL-C level increased significantly in BREE group. It is our investigation that these positive effects of BREE may be due to phenols, flavonoids and cyanidin-3 glucoside, because they are known to exert anti-oxidative effect (Halliwell, 2007; Cvorovic et al., 2010; Guo et al., 2008). These results agree with the results of Hou et al. (2010) who found that TG and TC levels were increased in serum of rats after administration of ethanol and were decreased in serum of rats after administration of black rice anthocyanin-rich extract. TG, TC and LDL were decreased significantly ( $P \leq 0.05$ ) and HDL was significantly higher ( $P \leq 0.05$ ) in plasma of mice fed with black rice extract diet (Chiang et al., 2006).

#### Effect of BREE on TBA, SOD, GSH and F2-isoprostanes activities in serum

Levels of TBA, SOD, GSH and F2-isoPs changes in PC and NC group rats and in rats after administration of EA (6 g/kg bw) + BREE (125 and 250 mg/kg bw) are as shown in Table 4. Data indicated significant ( $P \leq 0.05$ ) increase in the levels of TBA and F2-isoPs (19.68 nmol/L and 0.6991 pg/ml), respectively in PC as compared to NC (6.60 nmol/L and 0.2532 pg/ml), respectively. Treatment with ethanol results in the hepatic accumulation of MDA and decrease in antioxidant are increased oxidative stress and free radical mediated tissue damage in rats

**Table 4.** Effect of BREE on TBA, SOD, GSH and F2-isoprostanes activities in control and ethanolic administrated rats.

Treatment	TBA (nmol/L)	SOD (U/mL)	GSH (nmol/ml)	F2-isoP (pg/ml)
NC	6.60 <sup>d</sup> ±0.11	25.56 <sup>b</sup> ±0.20	28.77 <sup>b</sup> ±0.07	0.2532 <sup>c</sup> ±0.001
PC	19.68 <sup>a</sup> ±0.22	15.10 <sup>d</sup> ±0.07	12.10 <sup>d</sup> ±0.06	0.6991 <sup>a</sup> ±0.003
G1 (125 mg/kg bw BREE)	9.24 <sup>b</sup> ±0.11	22.54 <sup>c</sup> ±0.13	21.33 <sup>c</sup> ±0.10	0.3271 <sup>b</sup> ±0.002
G2 (250 mg/kg bw BREE)	7.90 <sup>c</sup> ±0.07	26.06 <sup>a</sup> ±0.10	29.04 <sup>a</sup> ±0.09	0.2513 <sup>c</sup> ±0.001
LSD	0.42449	0.40097	0.253	0.005

BREE, Black rice ethanolic extract; TC, Total cholesterol; HDL-C, high density lipoprotein; LDL-C, low density lipoprotein; Gand G2 Groups, 1 and 2. Values are mean (dev for 10 rats in each group. NC, Compared with negative control group:  $P \leq 0.05$ ; PC, Compared with positive control group:  $P \leq 0.05$  (One-way ANOVA followed by Fischer's LSD test).

and human (Minana et al., 2002). The reduced levels may be due to ROS being generated during alcohol metabolism that lead to lipid peroxidation and GSH oxidation.

The important tripeptide non-enzymatic antioxidant is GSH, which is thought to be an important endogenous defense molecule against peroxidative cellular destruction membranes. It reacts directly with electrophilic metabolites and ROS, serves as a substrate for several enzymes and prevents essential thiol groups from oxidation. In the present study, GSH concentration was reduced significantly in alcoholic-treated group rats (Table 4) which were in support with several reports (Jang et al., 2012; Chiang et al., 2006). Administration of black rice ethanolic extract with ethanol significantly altered the activities of both the non-enzymatic and enzymatic antioxidants to near the levels of normal group.

Interestingly, SOD and GSH were significantly ( $P \leq 0.05$ ) lower (15.10 u/mL and 12.10 nmol/ml) in PC rats, respectively than NC (25.56 u/mL and 28.77 nmol/ml), respectively. On the other hand, BREE (125 and 250 mg/kg bw) treated groups significantly ( $P \leq 0.05$ ) alleviate the TBA, SOD, GSH and F2-isoprostanes to near normal levels (Table 4). This decrease could be due to ROS inefficient scavenging which might be implicated to oxidative inactivation of enzymes (Jayaraman et al., 2009), which proves to be a potent antioxidant, a finding that correlates with recent reports (Chiang et al., 2006; Arulmozhi et al., 2012; Hsieh et al., 2008). Furthermore, the antioxidant property and the oxygen-radical scavenger of the extract may therefore be due to the presence of polyphenolic high content compounds such as anthocyanins and flavonoids (Mira et al., 2009; Yawadio et al., 2007; Zhang et al., 2010). Hou et al. (2010) found that ethanol treatment caused a severe increase in serum MDA and decrease in GSH concentration ( $P \leq 0.05$ ) in rats, while, black rice anthocyanin-extract reduced the formation of serum MDA and restored the levels of non-enzymatic antioxidant in tissues. Lieber et al. (1995) have reported increased hepatic F2-isoPs in liver biopsy specimens from alcohol fed baboons. Other investigators have reported increased

plasma F2-isoP levels in alcoholic hepatitis and cirrhosis patients (Lieber, 1997; Delanty et al., 1996; Aleynik et al., 1998).

## Conclusion

The results showed that BREE has a protective action against EA induced oxidative stress; this protective effect is mainly due to its antioxidant properties and free radical scavenging activity which has been suggested as a possible mechanism of action of BREE against ethanolic toxicity. It indicates the therapeutic values of black rice and their potential role in preventing cardiovascular diseases. This observation points to a new direction when trying to understand the physiological function of black rice extract as a benefit to human health.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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## Full Length Research Paper

# Production and purification of extracellular pullulanase by *Klebsilla aerogenes* NCIM 2239

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The present study was carried out to optimize the production of pullulanase from *Klebsilla aerogenes* NCIM 2239, a new strain of the bacterium. The organism was screened for its ability to synthesize and secrete extracellular pullulanase, by analysis of pullulan degrading activity. Environmental (pH and temperature) and nutritional (carbon and nitrogen) factors influencing growth and product formation were optimized through fermentation trials in shake flasks. Maximum pullulanase production (78.62 U/ml) was observed at 48 h. Optimized pH and temperature were 7.0 and 37°C, respectively. Different carbon sources (pullulan, starch, sucrose, maltose and glucose) and nitrogen sources (peptone, beef extract, yeast extract and casein) were analysed for maximum production of pullulanase. This bacterium was found to exhibit maximum pullulanase production using starch and peptone as carbon and nitrogen sources, respectively. In addition, pullulanase production by solid substrate fermentation (SSF) was investigated using *K. aerogenes* NCIM 2239. The extracellular pullulanase was purified by ultrafiltration method of membrane separation. The pullulanase activity after ultrafiltration was 130.21 U/ml when compared with its crude (83.08 U/ml).

**Key words:** Pullulanase,  $\alpha$ -1,6 glucosidic linkages, *Klebsilla aerogenes* NCIM 2239, solid state fermentation, ultrafiltration.

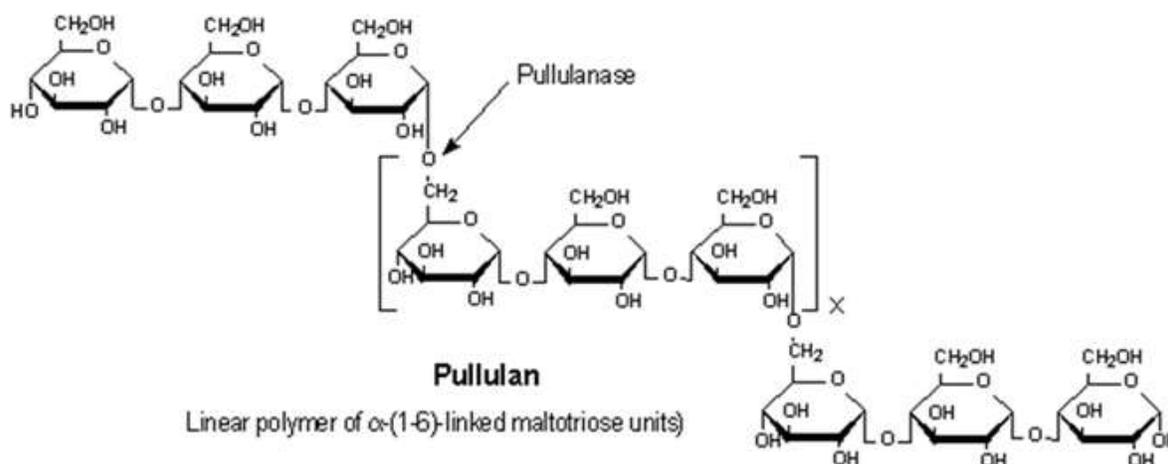
## INTRODUCTION

Pullulanase (pullulan  $\alpha$ -glucanohydrolase (EC 3.2.1.41) is an extracellular carbohydrase which was first discovered by Bender and Wallenfels in 1961 from mesophilic organism, *Klebsiella pneumoniae* (Murooka et al., 1989). Pullulanases are also called de-branching enzymes and have been widely used to hydrolyse the  $\alpha$ -1, 6 glycosidic

linkages in starch, amylopectin, pullulan and related oligosaccharides (Hii et al., 2012; Duan and Wu, 2015) (Figure 1).

Pullulan, a linear  $\alpha$ -glucan was synthesised by *Pullularia pullulan* (Bender and Wallenfels, 1961; Catley, 1971). It consists of repeating units of  $\alpha$ -maltotriose

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**Figure 1.** Pullulan degradation by pullulanase (Hii et al., 2012).

joined “head to tail” by  $\alpha$ -1, 6 bonds. Pullulanase specifically attacks  $\alpha$ -1, 6-glycosidic linkage of branched chains as well as  $\alpha$ -1, 6-glycosidic and  $\alpha$ -1, 4-glycosidic linkages within other polysaccharide residues; these properties have made pullulanase, a useful agent in structural studies of oligosaccharide and polysaccharide (Drummond et al., 1969).

A good number of pullulanases has been purified and characterized from different bacterial sources. Pullulanase type I has been characterized from mesophilic bacteria such as *Aerobacter aerogenes* (Ohba et al., 1973), *Bacillus acidopullulyticus* (Jensen et al., 1984), *K. pneumonia* (Bender and Wallenfels, 1961; Kornacker et al., 1990) and *Streptomyces* sp. (Takasaki et al., 1993). Moderate thermophilic gram positive bacteria such as *Bacillus flavocaldarius* (Suzuki et al., 1991), *Bacillus thermoleovorans* (Ben Messaoud et al., 2002), *Clostridium* sp. (Klingeberg et al., 1990) and *Thermos caldophilus* (Kim et al., 1996) also have the ability to secrete pullulanase type I, while pullulanase type I from hyperthermophilic bacterium, *Fervidobacterium pennavorans*, has also been reported (Koch et al., 1997). Unlike pullulanase type I, pullulanase type II is widely distributed among extreme thermophilic bacteria and Hyperthermophilic archaea (Sunna et al., 1997). The most thermostable and thermoactive pullulanase type II reported to date, was derived from hyperthermophilic archaeon, *Pyrococcus woesei* (Rudiger et al., 1995) and *Pyrococcus furiosus* (Brown and Kelly, 1993; Brown et al., 1990). The current study deals with the production of pullulanase from *Klebsilla aerogenes* NCIM 2239.

Pullulanase is of great significance due to its wide area of potential application. Pullulanase is widely used in industries in the saccharification of starch (Prakash et al., 2012). It converts starch into glucose and maltose which are used in the production of glucose syrup more efficiently. The products obtained by the action of

pullulanase have various pharmaceutical and nutraceutical properties. Pullulanase is inexpensive and easily available hence, it helps in the economical production of various products like resistant starch and substitutes of locust bean gum (LBG). The saccharification property of this enzyme has led to its use in various food industries for the production of glucose syrup, maltose syrup and beer (Shaw et al., 1992). Bioethanol production could also be done by the use of pullulanase thus providing a source of fuel. The basic property of breaking  $\alpha$  linkage has been widely used by the baking industry (Prakash et al., 2012).

The aim of this research work was to optimize the parameters of fermentation such as carbon sources, nitrogen sources, pH, temperature and incubation time for the enhancement of production of pullulanase from *K. aerogenes* NCIM 2239 and to purify the enzyme by ultrafiltration method. In addition, the effect of submerged fermentation and solid state fermentation was studied.

## MATERIALS AND METHODS

### Microorganism

The pullulanase-producing bacterium, *K. aerogenes* NCIM 2239, was procured from National Collection of Industrial Microorganisms (NCIM) and National Chemical Laboratory at Pune, India.

### Inoculum preparation

The strains were maintained by subculturing consecutively in maltose limiting medium at 37°C under aerobic conditions. For inoculum preparation, a loop from the stock was sub-cultured into the nutrient broth.

Single colony from the nutrient agar was inoculated into nutrient broth and incubated to obtain initial cell concentration. This culture was used as inoculum (1% v/v) for all fermentations carried out in this process.

### Production of pullulanase

Extracellular pullulanase secretion by *K. aerogenes* NCIM 2239 was obtained in a modified mineral Czapek medium (Hii et al., 2009) with maltose as a carbon source. The culture medium consisted of (w/v):  $K_2HPO_4$  (0.1%),  $NaNO_3$  (0.5%),  $MgSO_4 \cdot 7H_2O$  (0.05%), KCl (0.05%),  $FeSO_4 \cdot 7H_2O$  (0.001%), peptone (0.8%) and maltose (0.5%).

The pH of all media was adjusted to 7.0 and sterilized at 121°C for 20 min. After incubation of the cultures at 37°C for 2 days, the cells were removed from the culture medium by centrifugation at 10,000 rpm for 10 min.

### Determination of pullulanase activity

Enzyme activity was determined by measuring the enzymatic release of reducing sugar from pullulan. In this assay, 0.5 mL of enzyme sample was added into 0.5 mL of 1% (w/v) pullulan in 0.02 M sodium phosphate buffer at pH 7.0. The reaction mixture was incubated at 40°C for 30 min and the amount of reducing sugar released at the end of the reaction was determined by dinitrosalicylic acid (DNSA) method.

Sample blank was used to correct the non-enzymatic release of reducing sugar. One unit of pullulanase activity is defined as the amount of enzyme required to produce 1  $\mu$  mol reducing sugar (equivalent to glucose) per minutes under the assay conditions (Ling et al., 2009).

### Optimum conditions for pullulanase production

#### Effect of incubation period

A volume of 100 ml of selected medium was taken in each 250 ml conical flask. All flasks were autoclaved at 121°C at 20 min. After cooling, the flasks were inoculated. The flasks were incubated for 1 to 3 days. The cells were precipitated by centrifugation at 10000 rpm. From the supernatant, enzyme activity was measured.

#### Effect of incubation temperature

The culture medium was incubated at different temperature for optimum enzyme production. For this reason, equal quantity of inoculum was added in each conical flask containing 100 ml of selected suitable medium with selected pH.

The flasks were then incubated at different temperature (28, 37, 45 and 55°C), respectively, for optimum enzyme production. The effect of temperature on pullulanase production was recorded.

#### Effect of pH

To observe the effect of medium pH on enzyme production, 100 ml of selected medium at different pH (5, 6, 7, 8, 9 and 10, respectively) were used. The flasks were then inoculated and incubated in optimum incubation periods. The effect of medium pH on pullulanase activity was recorded.

#### Effect of carbon sources

The effect of carbon sources on pullulanase production was tested by using different carbon sources namely, sucrose, starch, pullulan, glucose and maltose. They were tested individually at the concentration of 1% in the carbon source-optimized medium.

### Effect of nitrogen sources

Nitrogen source is one of the most common factors which affect the pullulanase enzyme production. The organic nitrogen source used greatly influenced both growth and pullulanase production.

The growth medium was initially supplemented with different organic nitrogen sources (0.5%), that is, beef extract, peptone, yeast extract and casein. Then, the nitrogen source that gives maximum enzyme production was optimized.

### Solid state fermentation

10 g of rice bran, corn bran, rice bran + corn bran was taken and then,  $K_2HPO_4$  0.1 g,  $NaNO_3$  0.5 g,  $MgSO_4 \cdot 7H_2O$  0.05 g, KCl 0.05 g,  $FeSO_4 \cdot 7H_2O$  0.001 g and peptone 0.5 g were autoclaved and inoculated with 2 ml of 24 h activated bacterial suspension, incubated at 37°C for 48 h.

The enzyme was extracted by using 25 ml of distilled water to the solid substrate culture and well mixed by agitation for 5 min, then filtered through a Whatmann paper. The filtrate was centrifuged at 8000 rpm for 20 min. The supernatant was used as crude enzyme and the enzyme activity was assayed.

### Purification of pullulanase by ultrafiltration

The enzyme was purified by ultrafiltration method. Since the molecular weight of pullulanase was 98 kDa approximately (Malakar et al., 2012; Shehata et al., 2016), the polyether sulphone membrane of molecular weight cut-off 100 kDa was used.

In this method, the polyether sulphone membrane was fitted in membrane support. The experimental setup was placed above the magnetic stirring plates. In the feed reservoir, 150 ml of crude enzyme was loaded through the peristaltic pump and the pressure was passed for stirring.

Simultaneously, permeate was collected through an outlet (Liu et al., 2009). The collected volume of permeate was analyzed using DNSA method.

## RESULTS AND DISCUSSION

### Optimum conditions for pullulanase production

#### Effect of incubation period

The growth of microorganism and their enzymatic activities were estimated during incubation period which extended up to 48 h. The results are shown in Figure 2. The biosynthesis of extra cellular enzyme increased almost linearly until the stationary phase of growth was attained. Growth as well as pullulanase activity reached maximum value after 48 h of incubation.

The effect of incubation period was checked at an interval of 12 h till 72 h. The maximum enzyme activity was observed at 48 h after which the enzyme activity started to decrease. Similar result was reported from pullulanase enzyme from *Bacillus cereus* (Waleed et al., 2015).

The reason for this is the denaturation of the enzyme caused by the interaction with other components in the medium (Lonsane et al., 1990). It could have also been due to the fact that, the microorganism was on its decline

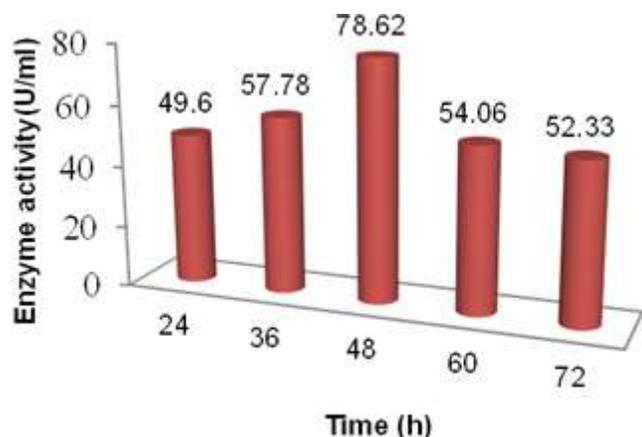


Figure 2. Effect of incubation time on pullulanase production.

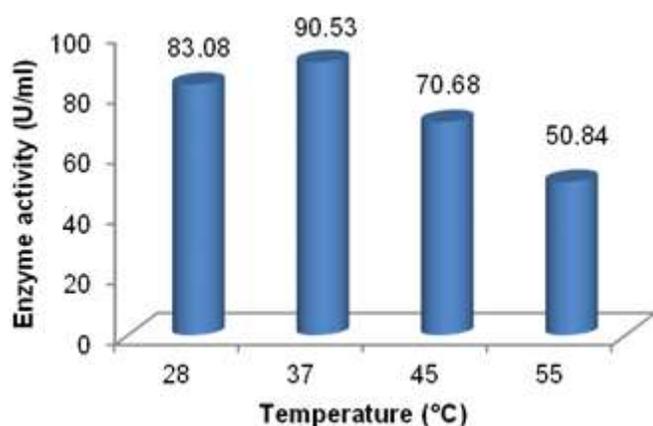


Figure 3. Effect of incubation temperature.

phase during the third day of fermentation which resulted in the decreased enzyme production (Figure 2). During long time exposure, proteins component would denature in the submerged fermentation medium.

### Effect of temperature

Temperature is one of the most important parameter governing the growth of organism and enzyme biosynthesis. The enzyme production pattern shows a gradual increase in the enzymatic activity giving maximum production at 37°C. Similar result was reported from pullulanase enzyme from *B. cereus* (Waleed et al., 2015).

At higher temperature due to the production of the large amount of the metabolic heat, the fermenting substrate temperature shoots up, thereby inhibiting microbial growth and enzyme production. The effect of temperature was observed in the range of 28, 37, 45 and 55°C. The

maximum enzyme activity was observed at 37°C after which, the enzyme loses its stability and activity decreases (Figure 3).

Similar results were reported for *Aspergillus niger* at 35°C (Lonsane et al., 1985), and for amylase production by *Bacillus* sp. at 37°C, others showed a high level of enzyme production from *Raoultella* which was 30°C (Hii et al., 2009). The optimum condition for amylopullulanase production by *Clostridium thermosulfogenes* was observed at 60°C (Swamy and Seenayya, 1996).

### Effect of pH

Microorganisms were grown at different pH in the range of 5 to 10, to determine the effect on pullulanase production. The degree of enzyme production was highest at pH 7 and reduces with the increase in pH as described in Figure 4. Similar result was reported from pullulanase enzyme from *B. cereus* (Waleed et al., 2015).

pH can influence the growth and product formation due to its effect on the solubility of nutrient, ionization of the substrate and its availability to the microorganism (Kotwal et al., 1998). Also, the acidic or basic media may lead to denaturation of the enzyme (Bertoldo et al., 2002).

An attempt to overcome the problem of pH variability during the solid substrate fermentation (SSF) process was obtained by the substrate formulation, considering the buffering capacity of the different components employed by the use of buffer formulation with components that have no deleterious influence on the biological activity (Kotwal et al., 1998).

### Effect of carbon sources

The effect of different carbon sources on the production of pullulanase was studied using the basal culture medium, supplemented with 1% of carbon sources. The culture containing 1% of each carbon source at a time was incubated and tested for enzymatic activity.

Among these glucose, maltose, pullulan, sucrose and starch were used as 1% at a time. Starch enhanced the pullulanase activity (92.76 U/ml) when compared with other carbon sources. Carbon source is the most effective factor for the production of pullulanase from microorganism.

In the presence of soluble starch, the production of pullulanase was increased as compared to other carbon sources. Maximum production of pullulanase was observed with soluble starch and hence, it is a good source of carbon used in production of pullulanase (Malviya et al., 2010).

The highest pullulanase activity may be due to inducible nature of maltose, being hydrolytic product of starch. Various carbon sources were checked as media component and pullulan was observed with best enzyme

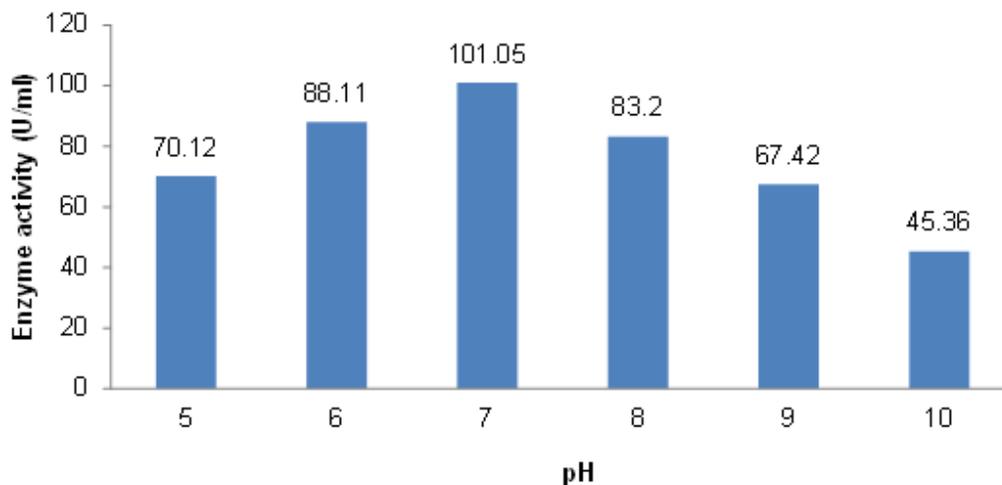


Figure 4. Effect of pH on pullulanase production.

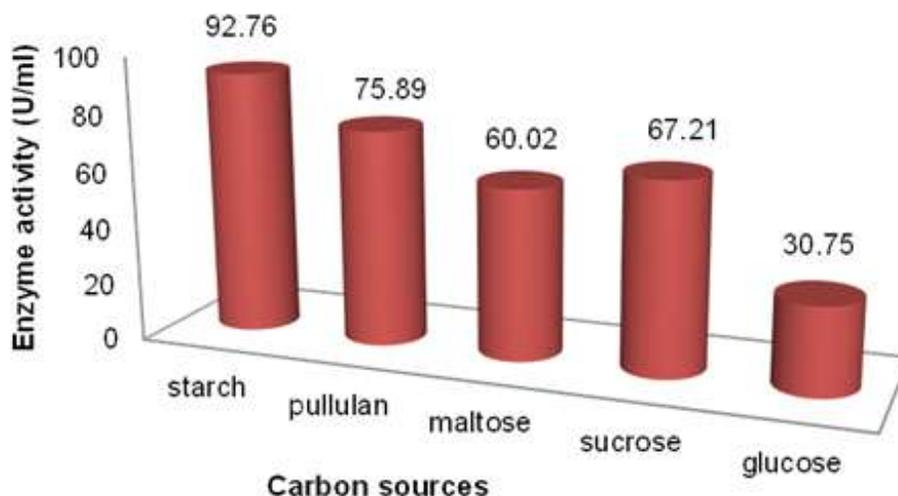


Figure 5. Effect of carbon sources on pullulanase production.

activity, while maltose, sucrose and glucose showed gradual decrease but significant in enzyme activity (Figure 5).

#### Effect of nitrogen sources

The effect of different nitrogen sources on the production of pullulanase was studied using the basal culture medium supplemented with 0.5% of nitrogen sources. Among the four organic nitrogen sources, peptone had more influence on pullulanase production (79.61 U/ml) than others (Figure 6). This may be due to the low molecular weight of peptone, which can be easily degraded and can be absorbed.

Different nitrogen sources were checked for enzyme

activity among which peptone gave the maximum activity. The highest value of pullulanase activity was achieved in medium containing peptone which indicates that, peptone could be one of favorable organic nitrogen source that enhances the cell growth of the culture as well as the pullulanase production (Hii et al., 2009).

#### Determination of optimal conditions for pullulanase production in submerged fermentation (SmF)

The result depicted in Table 1 showed that, media containing starch as carbon source with peptone as nitrogen source, was inoculated with 1 ml of bacterial suspension when incubated at 37°C for 48 h at pH 7, which was the best media for pullulanase production.

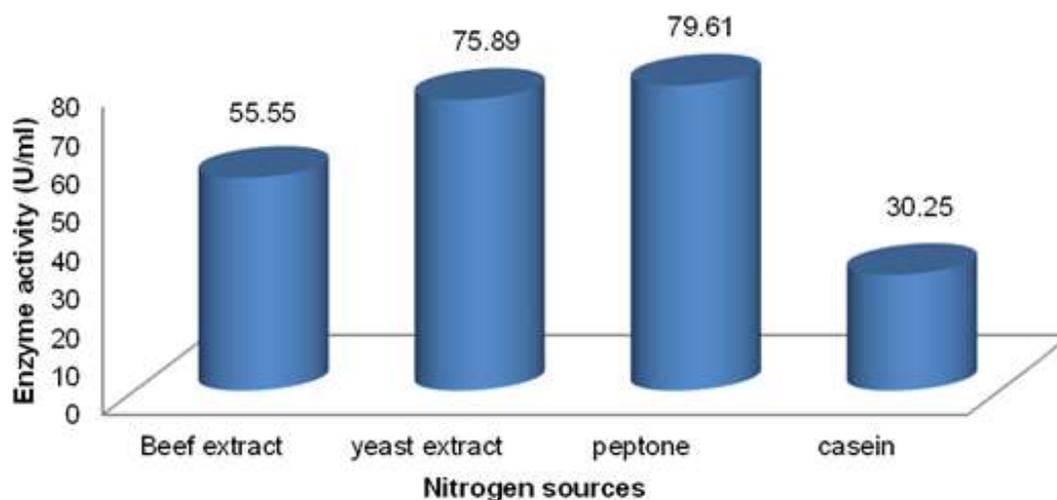


Figure 6. Effect of nitrogen sources on pullulanase production.

Table 1. Optimal conditions for pullulanase production by using submerged fermentation medium.

Parameters	Optimum for production	Enzyme activity (U/ml)
Carbon sources	Starch	92.76
Nitrogen sources	Peptone	79.61
Incubation period	48 h	78.62
Incubation temperature	37°C	90.53
pH	7.0	101.05

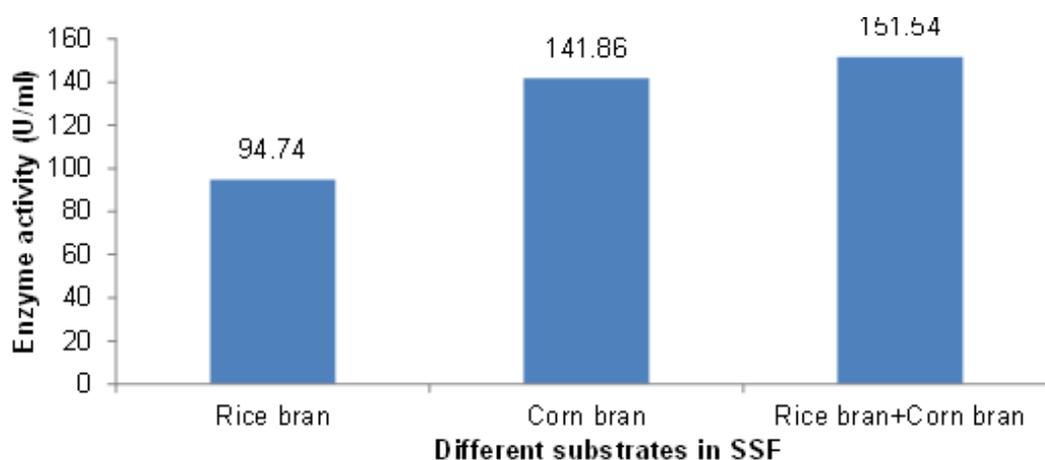


Figure 7. Enzyme activity of different sources in SSF.

### Solid substrate fermentation (SSF)

Different solid substrates were used for pullulanase production from *K. aerogenes* NCIM 2239, as shown in Figure 7, which gave the highest enzyme activity in media

containing mixture of rice bran and corn bran as substrate in SSF.

The enzyme activity reached 151.54 U/ml, while the media containing rice and corn bran gave the lowest enzyme activity. Similar results are also reported for

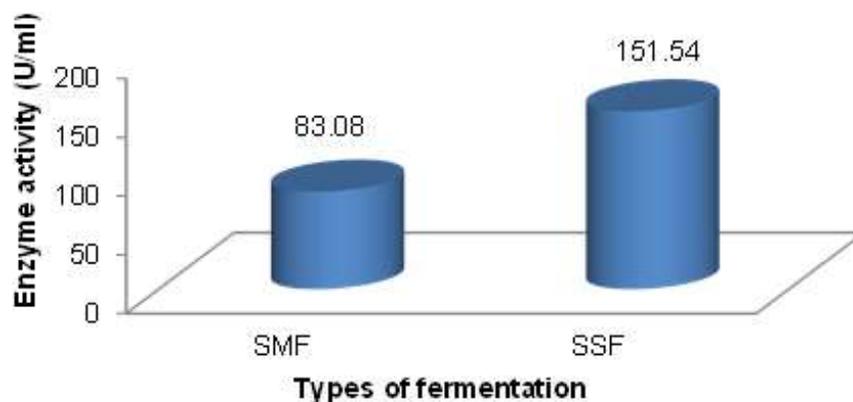


Figure 8. Comparisons between SSF and SmF.

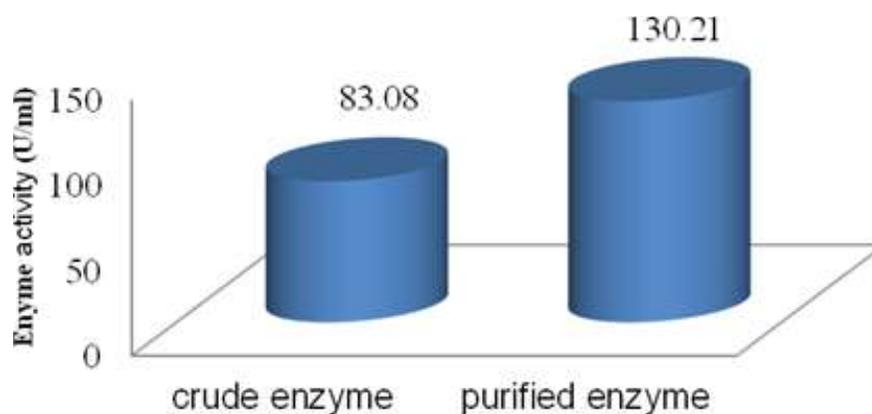


Figure 9. Enzyme activity between the crude and purified enzymes.

*Bacillus licheniformis* (BS18) (Khalaf and Aldeen, 2013).

### Comparison between SSF and SmF

SSF had given higher production of pullulanase than submerged fermentation (SmF). The results are shown in Figure 8.

SSF have been defined as the microbial transformation of biological materials in their natural state, in contrast with liquid or submerged fermentation that is carried out in dilute solution or slurries (Kotwal et al., 1998). The solid substrate in SSF provides a rich and complex source of nutrient, which may be sufficient or sometimes inadequate and incomplete, with respect to the overall nutritional requirement of that particular microorganism that is cultivated on that substrate (Kotwal et al., 1998). SSF is preferred to SmF because of the simple technique, low capital investment, lower levels of catabolic repression and end product inhibition, low waste output, better product recovery, and high quality production (Mahanta et al., 2008).

### Purification of crude enzyme by ultrafiltration method

The enzyme was purified by ultrafiltration method of membrane separation and enzyme activity, which was measured by DNSA method. The nature of solid substrate is the most important factor in SSF, which not only supplies the nutrient to the culture but also serves as an anchorage for microbial cells.

Therefore, the particle size and the chemical compositions of substrate are of critical importance (Kim et al., 1985) (Figure 9)

### Conclusion

Pullulanase was successfully produced and purified from *K. aerogenes* NCIM2239 strain. As it is very stable at higher range of temperature and pH, this can be used in many industrial processes. The media preparation, which is one of the most important objective of this study was achieved and the most appropriate combinations of media components were stabilised.

**Table 2.** Enzyme activity between the crude and purified enzymes.

Parameters	OD at 540 nm	Concentration ( $\mu\text{g/ml}$ )	Enzyme activity (U/ml)
Crude enzyme	0.32	498.51	83.08
Purified enzyme	0.51	781.25	130.21

In order to study enzyme activity, the enzyme substrate reaction was performed based on various parameters such as temperature ( $37^{\circ}\text{C}$ ), incubation time (48 h) and pH 7. Various carbon sources were checked as media component among which starch gave best enzyme activity, while maltose, sucrose and glucose showed significant enzyme activity.

Peptone gave the maximum activity among different nitrogen sources. The study of the SSF medium, SmF and comparison between them indicated that, the use of SSF gave higher production of enzyme than SmF. SSF technique is a suitable and economic method for pullulanase production. The pullulanase activity of the crude form was 83.08 U/ml. The crude enzyme under purification using ultrafiltration gave rise to 130.21 U/ml enzyme activity, and thus can be used in various industrial processes (Table 2).

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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## Full Length Research Paper

# Heterosis, heterobeltiosis, narrow-sense and broad-sense heritabilities for *Phytophthora megakarya* tolerance in two populations of *Theobroma cacao* L.

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A 2x2 diallel mating design was set up to estimate quantitative genetic parameters after leaf artificial inoculation tests in nursery and field for three consecutive years. F12 and F40 families of two reciprocal crosses viz., F12: ♀SCA12 × ♂ICS40 and F40: ♀ICS40 × ♂SCA12 were used to study heterosis (mid-parent), heterobeltiosis (better-parent), heritability (narrow-sense and broad-sense) and to quantify total polyphenols (TPP) and total flavonoids (TF) contents after inoculation of the leaves of parental and hybrid cocoa genotypes with *Phytophthora megakarya*. The results of heterosis and heterobeltiosis exhibited hybrid vigor of 86.66 and 85%, respectively. The highest heritability was identified [narrow-sense heritability ( $h^2$ : 0.668 for F12 and 0.682 for F40) and broad-sense heritability ( $H^2$ : 0.765 for F12 and 0.672 for F40)] for necrosis length in 2014. With regards to low narrow-sense heritability, it ranged from 0.019 to 0.162 while corresponding broad-sense heritability ranged from 0.476 to 0.562 for biochemical compounds. Among parent clones, ICS40 and SCA12 proved to have the general combining abilities (GCA). The use of these parental lines and understanding the gene flow of tolerance to black pod disease (Bpd) in cocoa will help researchers to develop a targeted breeding approach in generating new generation of tolerant genotypes cocoa.

**Key words:** Cocoa, heterosis, heterobeltiosis, heritability, black pod disease, tolerance.

## INTRODUCTION

Cocoa tree (*Theobroma cacao* L.) is a woody plant belonging to the Magnoliopsidae class. Because of its ecological requirements, cocoa farming has been largely

practiced in Africa (73% of world production, with 4.552 million tons), where four countries are among the five best producers in the world: Côte d'Ivoire (42%), Ghana

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(19%), Nigeria ranked behind Indonesia (7%), Ecuador (6.5%) and Cameroon (6%) (Anonymous, 2017). Although Cameroon is the fourth world cocoa producer, her cocoa crop is experiencing production setbacks due to black pod disease (Bpd) caused by an oomycete denominated *Phytophthora megakarya* (Nyasse, 1997).

Currently, disease control relies heavily on the use of chemical fungicides which has in turn increased the production costs and reduced growers' profits. In addition, there is a growing concern on the impact of chemical fungicides on the environment, which are costly and polluting for cocoa farmers. Genetic control based on hybridization allows the development and adoption of genotype tolerants to Bpd. This control strategy is the most preferred to improve production (Djocgoue et al., 2010; Ondobo et al., 2017).

Diallel cross analysis for a fixed set of populations provides a basis for preliminary determination of heterotic groups. Thus, knowledge on the nature and magnitude of genotypic and phenotypic variability present in the crop species plays a vital role in formulating a successful breeding program to evolve superior cultivars. Hence, heterosis per se is commercially useful.

However, heterosis (mid-parent) and heterobeltiosis (better-parent) are useful in shaping the direction that future hybrid breeding program should take. It also identifies the cross combinations which are promising in conventional breeding programs.

Studies on heterosis would help to generate breeding strategies of hybrid cocoa production. Obtaining genetic information from diallel cross progenies is a common practice of plant breeders working with allogamous crops.

However, the estimation of heritability serves as a useful guide to the breeder. By relying on heritability, the breeder is able to appreciate the production of the variation that is due to genotypic (broad-sense heritability) or additive (narrow-sense heritability) effects. If heritability of a character is very high, selection for the character should be fairly easy. Thus, estimates of heritability are useful in predicting the transmission of characters from the parents to their offspring (Parikh et al., 2016).

The present study was conducted firstly to estimate heterosis (mid-parent and better-parent) and heritability (broad-sense and narrow-sense) for necrosis length traits in a 2x2 diallel set of cocoa leaf and secondly to identify hybrids of *T. cacao* that are tolerant to Bpd and to observe good aptitudes for the combination of parent.

## MATERIALS AND METHODS

### Plant material

The experiment was conducted at the Cocoa Development Corporation (SODECAO) experimental field at Mengang Station. Two cocoa parental clones viz., SCA12 (moderately tolerant to Bpd and Forastero group) and ICS 40 (sensitive to Bpd and local

Trinitario group) were used to produce two reciprocal offspring populations (F12: ♀SCA12 × ♂ICS40 and F40: ♀ICS40 × ♂SCA12) by hand-pollination techniques for three seasons in three years (2012/2013, 2013/2014 and 2014/2015). These parents were crossed using 2x2 full diallel mating design. The parents were grafted in the nursery using bud wood.

### Plant inoculation

*P. megakarya*-lebdii isolate [obtained from the Central Laboratory of Phytopathology at IRAD (Research Institute for Agricultural Development)] was grown on V8 agar medium (20% V8 vegetable juice, 0.3% Ca<sub>2</sub>CO<sub>3</sub>, 1.5% agar and 1000 ml distilled water) and incubated in the dark at 25 ± 2°C.

Artificial inoculation of leaves of 22 genotypes was carried out in 2013, 2014 and 2015 (three-season period) adapted from Djocgoue et al. (2006). They were then inner surface sterilized with ethanol 70%. Three treatments were performed on each parental genotype and hybrid: (i) healthy, (ii) scarified (iii) and infected leaves. Agar disks (6 mm diameter) cut from 5-day-old fungal and straminopileous isolates were laid on the midrib after creating wounding with a sterilized razor blade. The scars were then covered with cotton that had been immersed in sterilized water. The necrosis length was measured at two days interval with a graduated ruler after inoculation. The samples were wrapped with aluminum foil every six days after inoculation.

### Estimation of heterosis and heterobeltiosis

The values of heterosis over mid and better parents from F<sub>1</sub> were calculated for length necrosis.

(i) Mid-parent heterosis (Zahour, 1992) was computed using the formula:

$$\text{MPH}(\%) = \frac{F_1 - \text{MP}}{\text{MP}} \times 100$$

(ii) Better-parent heterosis (or heterobeltiosis) was calculated using the procedure of Tang and Xiao (2013), following the formula below:

$$\text{BPH}(\%) = \frac{F_1 - \text{BP}}{\text{BP}} \times 100$$

In these formulas, MPH (%) represents the percentage of mid-parent heterosis, BPH (%) is the better-parent heterosis percentage, F<sub>1</sub> refers to the means of hybrid genotype, MP is the means of mid-parent and BP is the means of better-parent.

### Estimation of heritabilities ( $h^2$ and $H^2$ )

For necrosis length, two types of expression of heritability were calculated from formulas suggested by Falconer and Mackay (1996):

(i) Narrow-sense heritability ( $h^2$ ) measures the variance additive genetic.

$$h^2 = \frac{V_A}{V_P} = \frac{V_A}{V_G + V_E}$$

(ii) Broad-sense heritability ( $H^2$ ) measures any genetic variance.

$$H^2 = \frac{V_G}{V_P} = \frac{V_G}{V_G + V_E}$$

In these formulas,  $V_G$  is the genetic variance,  $V_P$  is the phenotypic variance,  $V_A$  is the additive genetic variance and  $V_E$  is the environmental variance.

### Extraction and total polyphenolic contents

The total polyphenolic (TPP) content was determined using the Folin-Ciocalteu method (Georgé et al., 2005) with some modifications. Leaves (0.5 g) were mixed with 2 ml of acetone/water (70:30, v/v) and incubated at room temperature. The mixture was centrifuged (Labofuge 400R) at 4500 rpm for 5 min. The supernatants were applied three successive washes with cyclohexane eliminate lipids, carotenoids and chlorophyll (Wieslaw et al., 1988). 2.5 ml of Folin-Ciocalteu (1:10) reagent was added to 0.5 ml of diluted crude extract (1:10) in a test tube. The mixture was incubated for 2 min in the dark and at room temperature. Then, 2 ml of  $\text{Na}_2\text{CO}_3$  (75 g/L) were added and the whole was stirred on a vortex and then incubated for 15 min at 50°C in a water bath in the dark. Afterwards, the mixture was cooled in an ice cube tray, the absorbance at 760 nm was immediately measured (Jenway 6305 spectrophotometer) against a blank in which the extract was replaced by distilled water. Results were expressed as gallic acid equivalents (GAE).

### Extraction of flavonoids

This was done following the extraction protocol described by Counet et al. (2004), with minor modifications. The plant material (cocoa leaves) lyophilized (50 mg) and crushed was extracted with 2 ml of the acetone/water/acetic acid (70:28:2 v/v/v). After upheaval at vortex, the mixture was incubated at room temperature for 1 h in the dark. The bottom was centrifuged (Labofuge 4000R) at 4500 rpm for 5 min and the supernatant was recovered and constituted the raw extracts. Raw extract undergone three successive washes with 1 x 3 ml of hexane in order to remove lipids, carotenoids and chlorophyll (Wieslaw et al., 1988).

### Total flavonoids contents

The total flavonoids (TF) content was determined using the aluminum trichloride ( $\text{AlCl}_3$ ) method (Ayoola et al., 2008). 2 ml of distilled water and 150  $\mu\text{l}$  of a 5% (w/v) sodium nitrite solution ( $\text{NaNO}_2$ ) were added to 500  $\mu\text{L}$  of diluted extract (1/10). The mixture was then incubated for six min in the dark and at ambient temperature ( $25 \pm 2^\circ\text{C}$ ). 150  $\mu\text{L}$  of the  $\text{AlCl}_3$  solution (10% in methanol; m/v) was added. After incubation for 6 min, at room temperature and in the dark, 2 ml of 4% (w/v) sodium hydroxide (NaOH) and 200  $\mu\text{L}$  were rapidly added to each tube. The reaction medium (5 ml) was homogenized before being incubated in the dark for 15 min, and the absorbance as recorded at 510 nm using a UV spectrophotometer (Jenway 6305). Quercetin was used for the construction of a standard curve and the results were expressed as mg quercetin equivalent (QE) per grams dry weight (DW) of plant material. The analyses were performed in triplicate.

### Statistical analysis

Data obtained from the observation were analyzed. SPSS (version

24.0 for windows) was used to perform analysis of variance (ANOVA). The significance of differences was determined by Tukey's multiple comparison technique. The Rho Spearman correlation was used to determine the correlation between all the parameters evaluated in the different treatments. Hierarchical classification and principal component analysis (PCA) of necrotic surface area data was performed with SPAD 5.5 software package.

## RESULTS AND DISCUSSION

### Length of necrosis

The development of necrosis for each of the three years: 2013, 2014 and 2015 was observed in parents (SCA12 and ICS40) and in hybrid populations F12 and F40 (Figure 1). However, individuals F12-36 [ $2.53 \pm 0.4$  cm (2013),  $2.26 \pm 0.42$  cm (2014) and  $5.93 \pm 0.31$  cm (2015)], F12-37 [ $1.98 \pm 0.01$  cm (2013),  $1.93 \pm 0.35$  cm (2014) and  $3.13 \pm 0.25$  cm (2015)] and F12-39 [ $2.70 \pm 0.21$  cm (2013),  $2.60 \pm 0.35$  cm (2014) and  $4.67 \pm 0.25$  cm (2015)] showed a decrease in the development of necrosis at the level of 2014 (Figure 1A). For this purpose, the same slides were observed in individuals F40-31 [ $4.03 \pm 0.21$  cm (2013),  $3.77 \pm 0.23$  cm (2014) and  $7.20 \pm 0.36$  cm (2015) and F40-34 [ $2.18 \pm 0.35$  cm (2013),  $2.17 \pm 0.32$  cm (2014) and  $5.40 \pm 0.26$  cm (2015)] (Figure 1B). However, F12-38 and F40-36 are considered highly susceptible to ICS 40 (moderately sensitive).

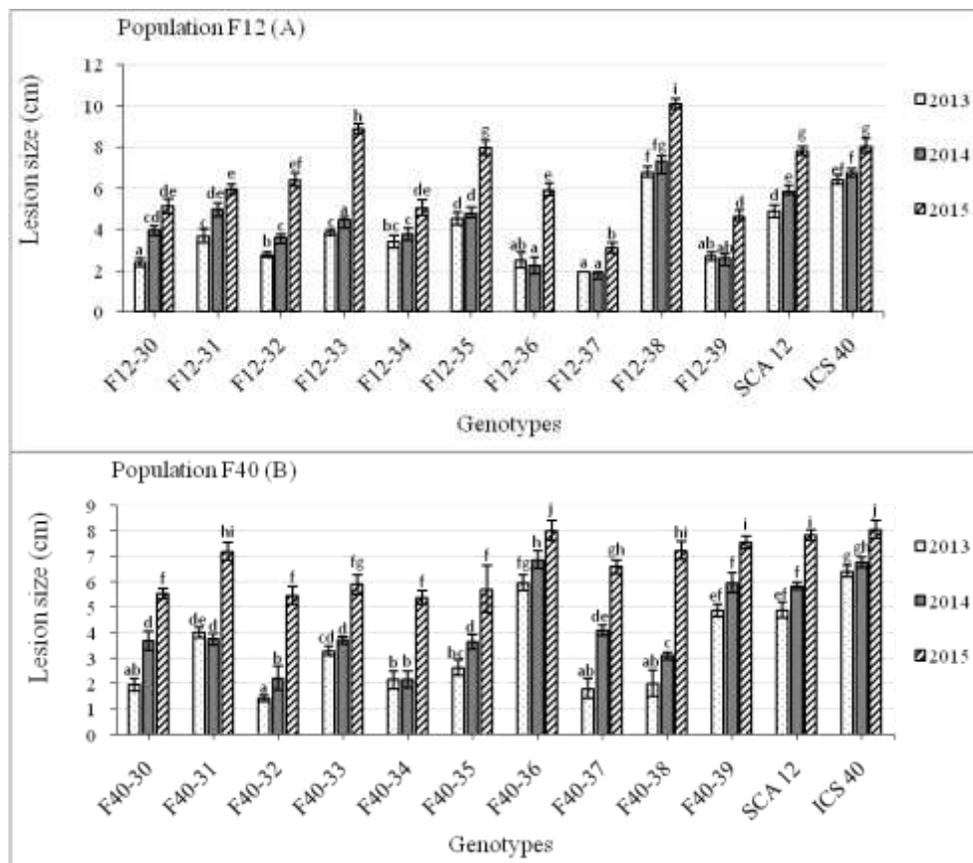
### Heterosis and heterobeltiosis

Six days after infection of the leaves, the hybrid F12 and F40 populations showed a positive heterosis effect of 86.66% (Table 1) on a continuous way (during the years 2013, 2014 and 2015). Likewise, genotypes F12-33 (+11.95) and F12-35 (+0.63), which showed a negative heterosis effect in the year 2013, on the other hand, they were again effective (positive heterosis) in the years 2014 and 2015.

Positive heterobeltiosis was manifested by a performance of hybrid genotypes superior to that of the better parent (SCA12). Only 85% of hybrid genotypes displayed negative values for the parameter studied after three years of evaluation (Table 1). Individuals F12-35 (-7.55) and F40-39 (-0.20) in 2015 showed that their positive heterobeltiosis is very small because their value was nearby to 0. Moreover, in hybrids F12-38 and F40-36 a continuously negative heterobeltiosis (periods 2013, 2014 and 2015) (Table 1).

### Broad-sense and narrow-sense heritabilities

Narrow-sense and broad-sense heritabilities estimates for necrosis character and biochemical parameters were obtained for the two populations from reciprocal crosses. Values of heritability in 2014 period were  $h^2 = 0.686$  and



**Figure 1.** Average lesion size (cm) of the midrib in two clones (SCA 12 and ICS 40) and their progenies F12 (A) and F40 (B) of *T. cacao* L., 6 days after inoculation with mycelium of *P. megakarya* during 2012/2013, 2013/2014 and 2014/2015 cocoa seasons. Values with the same letter for same genotype and in the same family are not significantly different ( $P < 0.05$ ). Values are means of 3 replicates.

$H^2 = 0.765$  for F12 and  $h^2 = 0.682$  and  $H^2 = 0.672$  for F40. These values were the highest of the other two years (2013 and 2015) (Table 2). Concerning (additive gene effects) and broad-sense (additive + dominance + epistasis effects), the values obtained in the (♀) SCA12 x (♂) ICS40 family ( $h^2 = 0.431$  and  $H^2 = 0.515$  for 2015) was not significant to that obtained in the (♀) ICS40 x (♂) SCA12 ( $h^2 = 0.301$  and  $H^2 = 0.576$  for 2015). The observation was the same for the 2013 and 2014 periods (Table 2).

### Principal component analysis (PCA) and hierarchical cluster

In the F12 family, the first two principal components (PC) generated from all data represented 98.15% of the total variability of necrosis during three years of study. The year 2015 was the dominant feature of the first axis [PC1 (91.45% of total variability)], while necrosis in the years 2013 and 2014 was characterized as the highest of the

second axis [PC2 (6.70% of total variability)] (Figure 2A).

The F40 family was dominated by necrosis in 2014 with 91.06% total variability, while necrosis progressed rapidly in the years 2013 and 2015 with a PC2 characterized by a total variability of 6.16% (Figure 2B). However, genotypes F40-36, F40-39, SCA12 and ICS40 which formed a group had higher necrosis sizes in all years 2013, 2014 and 2015.

Cluster analysis (5% dissimilarity) also classified genotypes by their affinities with the necrosis length character studied. The first cluster composed of genotypes that shared only 37% similarity among them. The second cluster consisted of two parents (SCA12 and ICS40) and eight hybrids correlated with 37.5% similarities. On the other hand, F12-38 (cluster 3) and F12-37 (cluster 4), differentiated themselves from other genotypes by their behavior (performance for F12-38 and vulnerable for F12-37) (Figure 3).

The ranking of parents and their offspring for necrosis length allowed classify more efficient genotypes other than the best parent "SCA12". F12, F40, families

**Table 1.** Heterosis and heterobeltiosis values (%) for length of necrosis of hybrid populations F12 and F40 over three years.

Genotype	MPH			BPH		
	2013	2014	2015	2013	2014	2015
<b>F12</b>						
F12-30	-35.43	-37.08	-57.63	-34.47	-32.14	-51.02
F12-31	-24.95	-20.76	-34.69	-23.83	-14.53	-24.49
F12-32	-19.08	-42.16	-50.75	-17.87	-37.61	-43.06
F12-33	+11.95	-28.84	-31.51	+13.62	-23.25	-20.82
F12-34	-36.27	-40.25	-39.45	-35.32	-35.56	-30.00
F12-35	+0.63	-23.93	-20.04	+2.13	-17.95	-7.55
F12-36	-25.37	-64.18	-55.34	-24.26	-61.37	-48.37
F12-37	-60.59	-69.41	-65.05	-60.00	-67.01	-59.59
F12-38	+27.04	+16.16	+20.04	+28.94	+25.30	+38.78
F12-39	-41.30	-58.80	-52.34	-40.43	-55.56	-44.90
<b>F40</b>						
F40-30	-30.40	-41.36	-65.23	-29.36	-36.75	-59.80
F40-31	-9.43	-40.25	-28.86	-8.09	-35.56	-17.76
F40-32	-31.24	-64.66	-74.76	-30.21	-61.88	-70.82
F40-33	-25.79	-40.89	-41.75	-24.68	-36.24	-32.65
F40-34	-32.08	-65.45	-61.69	-31.06	-62.74	-55.71
F40-35	-27.88	-42.16	-53.57	-26.81	-37.61	-46.33
F40-36	+1.05	+8.72	+5.38	+2.55	+17.26	+21.84
F40-37	-16.98	-34.71	-67.70	-15.74	-29.57	-62.65
F40-38	-9.01	-50.71	-64.17	-7.66	-46.84	-58.57
F40-39	-4.82	-5.23	-13.68	-3.40	+2.22	-0.20
<b>PHe (%)</b>	<b>80</b>	<b>90</b>	<b>90</b>	<b>80</b>	<b>85</b>	<b>90</b>

MPH: Mid-parent heterosis, BPH: better-parent heterosis, PHe: positive heterosis.

**Table 2.** Narrow-sense ( $h^2$ ) and broad-sense ( $H^2$ ) heritabilities values for the length of necrosis after three consecutive years of study.

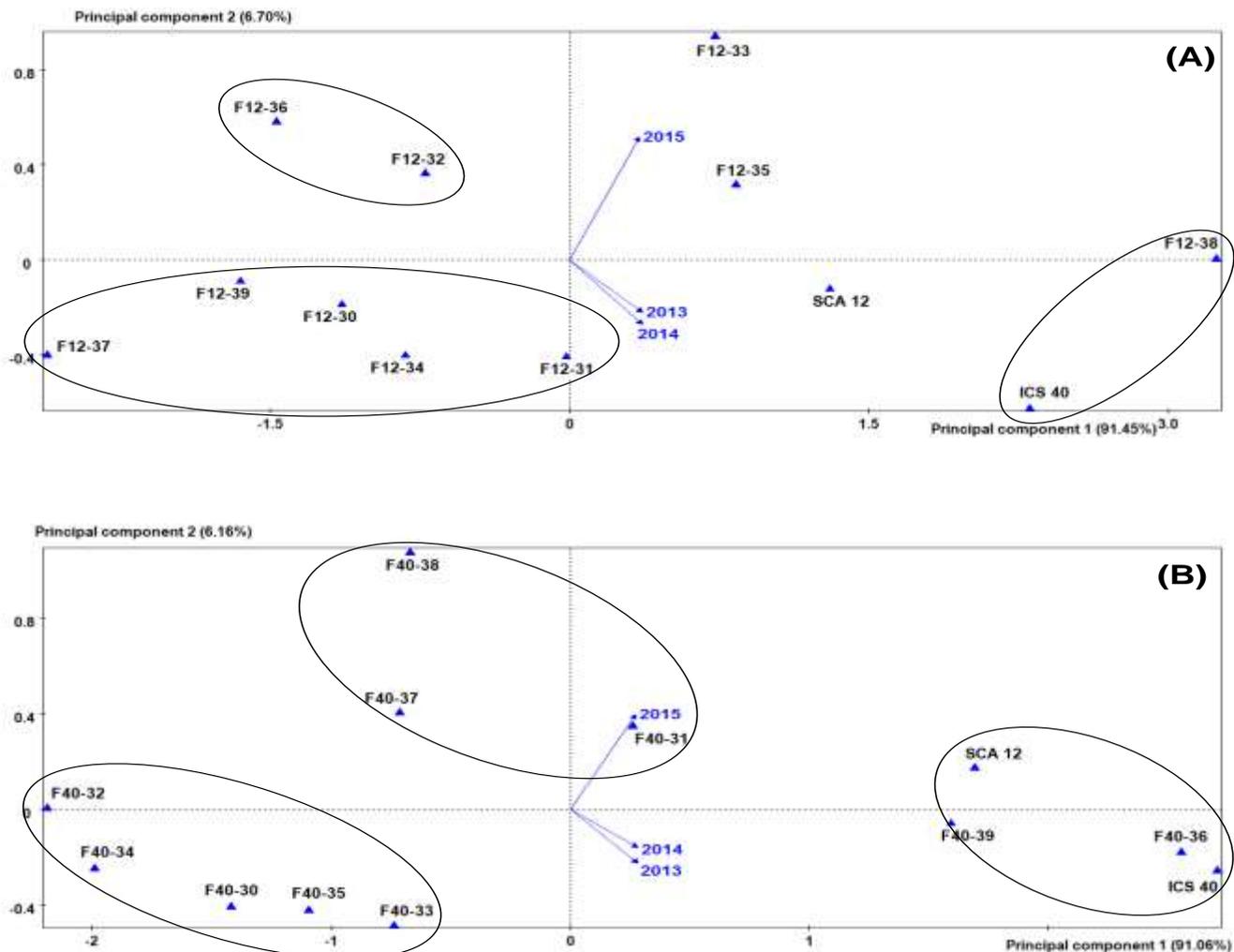
Reciprocal crossings	2013		2014		2015	
	$h^2$	$H^2$	$h^2$	$H^2$	$h^2$	$H^2$
F12: (♀) SCA 12 x (♂) ICS 40	0.419	0.466	0.668	0.765	0.431	0.515
F40: (♀) ICS 40 x (♂) SCA 12	0.412	0.514	0.682	0.672	0.301	0.576

respectively presented 7 (F12-37 > F12-39 > F12-36 > F12-30 > F12-32 > F12-34 > F12-31) and (F40-32 > F40-34 > F40-30 > F40-35 > F40-33 > F40-38 > F40-37) genotypes with the susceptibility level less than the best parent (Table 3). Intermediate genotypes with the susceptibility level less than the sensitive moderately parent "ICS40" presented 2 (F12-33 > F12-35) and 2 (F40-31 > F40-39) (Table 3).

### Phenols and flavonoids

Biochemical component in the leaves of hybrids and

parents inoculated with *P. megakarya* (Figure 4) showed significant variations among the healthy, scarified, scarified and inoculated, non-inoculated and parents for total polyphenols and flavonoids content between tolerant and susceptible hybrids. Tolerant hybrids, F12-37 and F40-32, had accumulated relatively higher TPP (5.44 and 11.91 mg EAG/g DW) and TF content (2.63 and 7.53 mg EAG/g DW), respectively. This was contrary to susceptible hybrids recorded low content (Figure 4). However, Table 4 shows the heritability values in the narrow-sense [ $h^2$  was low (varying between 0.019 to 0.063)] and in the broad-sense [ $H^2$  was high (ranging from 0.476 to 0.562)] of the accumulation of biochemical



**Figure 2.** Principal component analysis based on the development of cocoa leaf necrosis between 2013 and 2015 of parental and hybrid genotypes of F12 (A) and F40 (B) reciprocal populations.

compounds of two reciprocal populations.

### Correlation coefficients

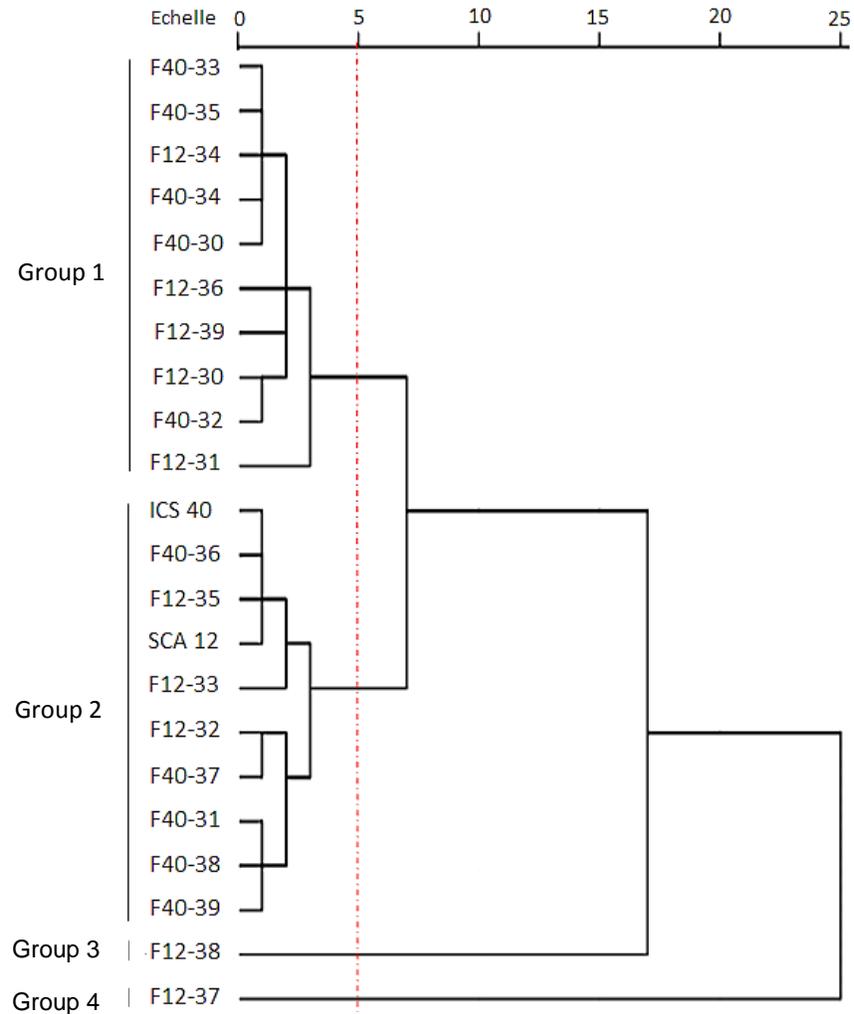
The trials that were carried out revealed significant correlation in the two families studied. The significant negative correlation was noticed between the necrosis length and biochemical component [F12: TPP ( $r = -0.959$ ,  $P < 0.05$ ) and TF ( $r = -0.944$ ) and F40: TPP ( $r = -0.886$ ,  $P < 0.01$ ) and TF ( $r = -0.771$ ,  $P < 0.05$ )] (Table 5). Besides, a significant positive correlation between TPP and TF (F12 and F40 populations,  $P < 0.05$ ) was recorded (Table 5).

### DISCUSSION

In this study, we attempted to estimate heritability

(narrow-sense and broad-sense), heterosis (mid-parent and better-parent) and understand the role of metabolites in the interaction *T. cacao* L./*P. megakarya*. The length of necrosis and the biochemical compounds (TPP and TF) were determined in the leaves of the parental and hybrid genotypes of *T. cacao* L. In order to achieve this objective, the production of the plant material (grafting and hand-pollination) was the fundamental element of this work.

The experiment and field work took three consecutive years of assessment of the tolerance level of F12 and F40 populations in nurseries and fields. Necrotic lesion size was observed 48 h after inoculation of the fungal mycelia. This confirmed that this duration is necessary for the interaction between the two entities and the beginning of resistance of cocoa tissues. The length of necrosis was favored by the vascularization of midrib which facilitates the progression of the disease. These results have been already obtained by Effa et al. (2016)

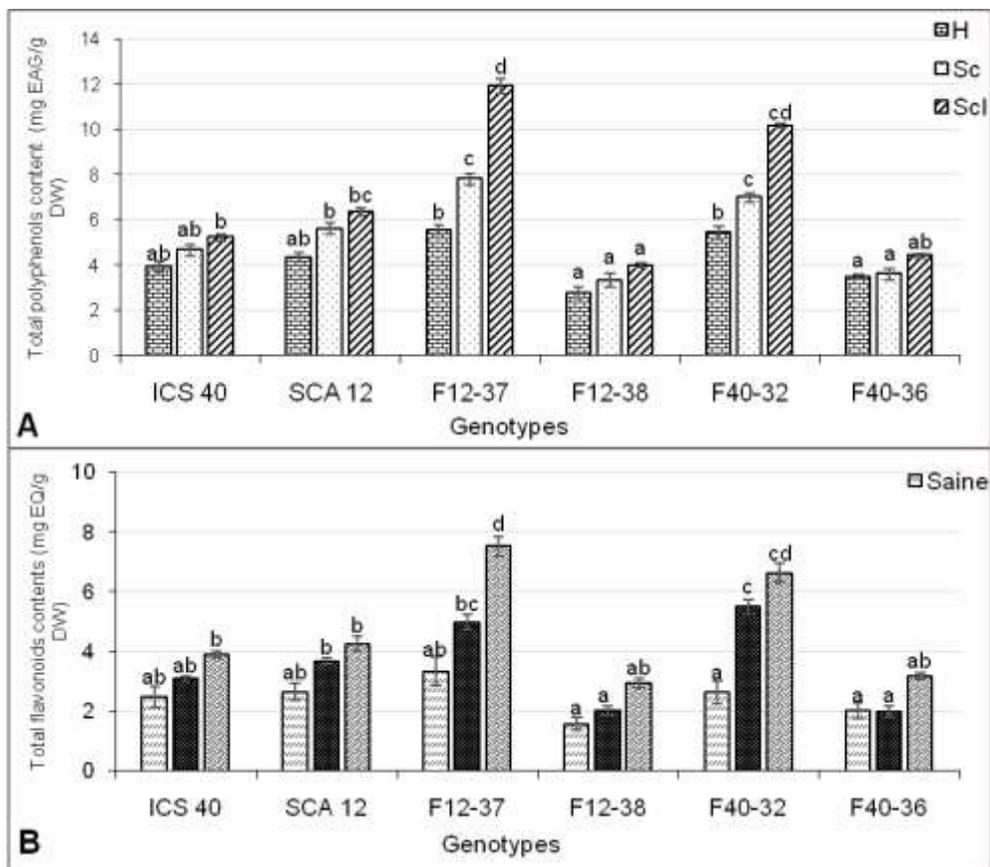


**Figure 3.** Dendrogram of the F12-F40 populations and their parents, SCA 12 and ICS 40 based on the length of necrosis of the years 2013, 2014 and 2015.

**Table 3.** Classification of the different parents and their progenies according to their susceptibility to black pod disease.

	<b>F12: (♀) SCA12 x (♂) ICS40</b>	<b>F40: (♀) ICS40 x (♂) SCA12</b>	
- Sensitive	F12-37 <sup>d</sup>	F40-32 <sup>d</sup>	
	F12-39 <sup>cd</sup>	F40-34 <sup>d</sup>	
	F12-36 <sup>cd</sup>	F40-30 <sup>cd</sup>	
	F12-30 <sup>cd</sup>	F40-35 <sup>cd</sup>	
	F12-32 <sup>cd</sup>	F40-33 <sup>cd</sup>	
	F12-34 <sup>c</sup>	F40-38 <sup>cd</sup>	
	F12-31 <sup>c</sup>	F40-37 <sup>c</sup>	
	<b>SCA 12<sup>bc</sup></b>		
	F12-33 <sup>b</sup>	F40-31 <sup>b</sup>	
	F12-35 <sup>b</sup>	F40-39 <sup>b</sup>	
	<b>ICS 40<sup>a</sup></b>		
+ Sensitive	F12-38a	F40-36 <sup>a</sup>	

1, Fourteen tolerant hybrids; 2, four intermediate tolerant hybrids. \*Values with the same letter in the same column and in the same family are not significantly different (Tukey's test,  $P < 0.05$ ).



**Figure 4.** Variations of total polyphenol (A) and flavonoids (B) content in three conditions of treatment (H=Healthy, Sc=scarified and Scl= scarified and inoculated). Values with the same letter in the same family are not significantly ( $P < 0.05$ ) different. Values are means of 3 replicates.

**Table 4.** Narrow -sense ( $h^2$ ) and broad-sense ( $H^2$ ) heritabilities values of the TPP and TF content of the four reciprocal crosses.

Reciprocal crossings	$h^2$		$H^2$	
	TPP	TF	TPP	TF
1 F12 : (♀) SCA 12 × (♂) ICS 40	0.155	0.063	0.562	0.531
2 F40 : (♀) ICS 40 × (♂) SCA 12	0.162	0.019	0.477	0.476

**Table 5.** Coefficients of correlation between tolerance tests and biochemical compounds of populations F12 and F40.

F12	Necrosis	TPP	TF
Necrosis	1	-0.959*	-0.944
TPP		1	0.973**
TF			1
F40	Necrosis	TPP	TF
Necrosis	1	-0.886**	-0.771*
TPP		1	0.941
TF			1

\*Correlation is significant at the 0.05 level; \*\*correlation is significant at the 0.01 level.

on the leaves attached to the plant (cocoa) in nursery and field. This result supports the findings of Nana et al. (2011), after the evaluation of necrotic lesions on cocoa pod cortex (SNK10 and SNK413).

Heterosis [mid-parent (86.66% for F12-40)] and heterobeltiosis [better-parent (85% for F12-40)] were evaluated for the development of necrosis character during three cocoa seasons (2013, 2014 and 2015), exhibited hybrid vigor, indicating good general aptitude for the combination of parents in reciprocal crosses (F12: ♀SCA12 × ICS40, F40: ♀ICS40 × ♂SCA12). From this, it is therefore suggested that these individuals demonstrate hybrid vigor, which would imply the presence of the additive and dominant gene effect in the transmission of character (Djocgoue et al., 2006). The results of the present investigation can be linked to those obtained previously by Manga et al. (2016). Such results show that tolerance to *P. megakarya* is controlled by the action of additive, dominant and epistatic genes. However, the role of dominant gene effect is more significant than that of additive gene effect. These observations corroborate those obtained by Ondobo et al. (2017) on the cocoa tree. Mohammadi et al. (2011) working on wheat, showed that the involvement of genes with additive and dominant effects in the estimation of heterosis (HPM and HMP) could be important in the selection of varieties.

These results matched with those of Djocgoue et al. (2010) and Manga et al. (2016), which showed a variation of strongly inheritable characters ( $h^2 > 0.4$ ). The work of Ram and Jabeen (2016) have shown that high values in a narrow sense heritability for a particular trait indicate that genes are largely controlled in an additive model in *Abelmoschus esculentus* L. Moench and *Zea mays* L., respectively. In the studied character, the absence of a significant difference between the heritability values from reciprocal crossing portrays the absence of maternal heritability. This finding suggested that the transmission of this character would not be cytoplasmic but nuclear (Ondobo et al., 2017). In fact, nuclear transmission of characters involved genetic additive effects. Recent studies by Parikh et al. (2016) showed that additive genetic effects are more important than non-additive effects (dominance and epistasis) for powdery mildew resistance in wheat.

The high heritability values reported in this study suggest that most of the phenotypic variance in Bpd resistance observed in the two reciprocal crosses is attributable to genetic effects. Similar results were observed for quantitative traits in tobacco (Aleksoska and Aleksoski, 2012), where it was concluded that high broad-sense and narrow-sense heritability were indicative of traits being controlled by genetic factors as opposed to environmental effects. Estimates of heritabilities for broad-sense and narrow-sense are useful tools to predict the genetic gain resulting from a specific reciprocal crossing. However, general combining abilities (GCA) are predominant for intra- and inter-family selections. This

means that the tolerance character is essentially transmitted additively (Ondobo et al., 2017).

Principal component analysis (PCA) and dendrogram based on necrosis length was used to categorize all the families; each group consists of similar individuals characterized by low and high length of necrosis. The tolerant hybrids characterized by low and intermediate length of necrosis were considered as elites. This result confirms a good aptitude (cross) towards parental gene combinations (Cilas et al., 2004).

Plant biochemical constituents have received considerable attention in relation to disease resistance. Most plants synthesize toxic compounds such as phenols and flavonoids during normal development, and their role in the resistance mechanism has been reported earlier by many authors (Nyadanu et al., 2013; Manga et al., 2016). These can be used as markers for selection of tolerance genotypes. Tolerant plants, when subjected to biotic stress, showed elevated levels of free phenolics and contained more lignin. Their role in resistance mechanisms was previously reported by Effa et al. (2016). These results are consistent with those of Ondobo et al. (2017), which emphasize that these compounds act as barriers against pathogen invasion and hence constitute part of host resistance mechanisms. This accumulation of flavonoids supported the assertion that these compounds are a major class of polyphenols involved in the resistance of cocoa against *P. megakarya*. Among these flavonoids, flavan-3-ol units are a major class with regards to the reaction of cocoa to stress (Effa et al., 2016). This is supported by the findings of Djocgoue et al. (2007) who reported that qualitative analysis of phenolics in leaves of cocoa showed a higher accumulation of the flavonoids (luteolin and apigenin derivatives) and some hydroxycinnamic acid derivatives.

The significant negative correlation between metabolites (TPP and TF) and length of necrosis trait reflects the importance of the biochemical compounds in tolerance expression. The findings indicate that TPP and TF play a role in disease resistance against *P. megakarya*. The results support the findings of many authors. Ngadze et al. (2012) disclosed that potato varieties with a high content of these compounds in tuber tissues can exhibit tolerance to pathogen attack. Nyadanu et al. (2013) suggested that total polyphenols, soluble sugars, insoluble sugars, nitrogen, proteins, flavonoid, tannins and lignin are involved in resistance of cocoa to black pod disease caused by *P. palmivora* and *P. megakarya*.

## Conclusion

The overall evaluation of 20 genotypes cocoa hybrids led to identification of the tolerant hybrids with about 85.83% of hybrid vigor. Narrow-sense heritability for Bpd tolerance observed in the current study indicates that

additive gene effects are more important than non-additive effects (dominance and epistasis) for Bpd tolerance.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

## ACKNOWLEDGEMENT

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