

## Phytochemical screening and quantitative variation of some secondary metabolites in five cultivated rice varieties

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### ABSTRACT

*Objectives:* Rice is an important food crop in West Africa whose production is known to be limited by biotic and abiotic constraints. Plants contain many chemical compounds beneficial for the control of pests. This study aims to compare the secondary metabolites profile and content in five rice varieties in relation with their resistance against pests.

*Methodology and results:* This research makes qualitative and quantitative assessment of secondary metabolites present in leaves of five rice varieties (WAB56-104, ITA306, CG14, TOG5681 and RAM55) using standard methods. Phytochemical screening revealed the absence of alkaloids and the presence of many other molecules including reducing sugars and phenolic compounds. Some other molecules were noticed in specific varieties such as catechetic tannins (variety CG14), terpenoids (variety WAB56-104) and saponosides (variety CG14). Quantitative estimation revealed that total phenols, flavonoids and tannins contents varied significantly among varieties whereas total anthocyanins content was similar in the five varieties tested.

*Conclusions and applications of findings:* The present study revealed that rice leaves contain various types of secondary metabolites mainly in the groups of phenol (tannins and flavonoids), mucilages, heterosides and reducing compounds. There is a high variability of secondary metabolites profile and contents between rice varieties. Varieties WAB56-104, RAM55 and ITA306 contained more phenols, more flavonoids and more tannins than the others did. These results confirm the importance of secondary metabolites in plants and suggest that the presence and/or the concentration of a specific metabolite could play a key role in the resistance of rice against insects. The present findings could be used to establish the relationship between secondary metabolites profile/concentrations and the resistance to specific pest in rice varieties.

**Keywords:** Terpenoids, phenolic compounds, reducing sugars, alkaloids, heterosides, mucilages, rice, resistance, insects

## INTRODUCTION

Rice (*Oryza* sp.) is an important food crop in West Africa (Blein *et al.*, 2008). There is a remarkable increase in rice consumption in this region due to demographic growth, and increase in urbanization and (Africa Rice Center, 2007). In addition, the per capita consumption increased progressively; from 32 kg in 1990 to 49 kg in 2012 (USDA, 2014). Domestic rice consumption is increasing at a rate of 8% per annum, surpassing domestic rice production growth rates of 6% per year. The production-consumption gap in West Africa is being filled by imports that are estimated at over US\$ 1.4 billion per year (Somado *et al.*, 2013). According to those authors, this gap is due to biotic and abiotic constraints. The main abiotic stresses affecting rice production in West Africa are drought and salt stress (Manneh *et al.*, 2007; Bocco *et al.*, 2012). Among biotic stresses, insects are the most important. Rice is affected by about 50 species of insects from two families that are Lepidoptera and Diptera. The wide frequent Lepidoptera are *Chilo suppressalis*, *Maliarpha separatella*, *Sesamia calamistis*, *Nymphula depunctalis*, *Eldana saccharina* and *Scirpophaga innotata*. The wide frequent Diptera are *Diopsis* spp. and *Orseolia oryzivora*. Those species are defoliators or stem borers causing the worst damage, as they infest rice plants from the seedling stage to maturity. The main control measures are crop management practices, genetic and chemical control (Wopereis *et al.*, 2008). Producers use large amounts of pesticides to increase rice production by protecting plants from insects damages (Nwilene *et al.*, 2006). For insect control, less effort has been made in the chemical investigations on rice plant species in the recent time (Balick *et al.*, 1996). Plants produce active

phytochemical molecules such as alkaloids, anthraquinones, flavonoids, phenols, saponins, steroid, tannins, terpenes etc (Namukobe *et al.*, 2011). Phytochemicals are non-nutritive compounds (secondary metabolites) found in plants to protect them against pests. They also contribute to flavor and color of a plant (Molefe-Khamanga *et al.*, 2012). Previous studies in Nigeria revealed that only, *Oryza glaberrima* Steud, cultivars of the traditional African rice species, have a high resistance to African Rice Gall Midge (AfRGM) (*Orseolia oryzivora*). However, no resistant cultivar has been found against the AfRGM among the *Oryza sativa*, an elite Asiatic rice species (Omoloye *et al.*, 2002). Omoloye *et al.* (1998) and Omoloye *et al.* (2002) showed that the host rice defense mechanism against the AfRGM, particularly in the resistant *O. glaberrima* cultivars, is mainly antibiosis, in which biochemical responses play a main role. For instance, phenols are precursors of lignin and suberin, which act as physical barriers against invasive pests (Marschner, 1995). Similarly, steroids regulate plant growth and development (Fang & Badger, 2003; Fujioka *et al.*, 2006), and can also influence and direct the feeding behavior of insects (Behmer & Elias, 2000; Yang *et al.*, 2001). Specific sterols in the preferred host plants are obtained and used by insects as precursors in the synthesis of hormones and as essential components of the insect cell membrane (Svoboda, 1984). Thus, the objectives of this study were to determine the secondary metabolites present in five rice varieties and assess the role of biochemical qualities and quantities in controlling rice insect pests.

## MATERIAL AND METHODS

**Plant material:** Five leaves samples from elite rice varieties were drawn through screening for secondary metabolites. These varieties are CG14, RAM55 and TOG5681 belong to *Oryza glaberrima* (African traditional rice species) while WAB56-104 and ITA306 belong to *O. sativa* (Asiatic rice species).

**Experimental sites:** Rice plants were grown on the farm of Biocontrol Center of IITA-Benin, Cotonou-station (06°25.415N, 02°19.684E and 21m altitude, 12km North-West from Cotonou) at Togoudo in Southern Benin. The station is located in the African coastal Savannah zone, with a subequatorial climate and hydromorphic soil (Adam and Boko, 1993). The

biochemical study was carried out at Laboratory of Chemistry and Organic Pharmaceutical in the Faculty of Health Sciences at University of Abomey-Calavi in Cotonou.

#### **Phytochemical analysis**

**Collection and preparation of plant material :** The leaves used were collected before the plant flowering stage and processed into powder. Firstly, the leaves were dried at room temperature for 15 days. Secondly, the dried leaves were ground into fine powder using mechanical grinder. At the last, the powder was packed in airtight container to control for humidity and then stored at room temperature.

**Phytochemical screening :** The phytochemical screening of leaf powders was assessed using the method described by Houghton and Roman (1998). Phytochemical screening was carried out on the leaf powders to identify the major natural chemical groups. The groups include phenolic compounds (anthocyanins, tannins, flavonoids, coumarins); essential oils or terpenoids (triterpenoids, sterols); heterosides (saponins, glycosides, heterosides, cardiac glycosides, derived cyanogenic, derived quinines, free antracenic); alkaloids; reduced sugars and mucilages).

**Phenolic compounds determination protocol:** The various extracts were obtained by stirring 50 g of powder per sample into 500 ml of a methanol-water mixture (70:30; v/v) with 0.5% of formic acid. Each mixture was homogenized for one hour with sonicator (Bandelin Sonorex Super RK255H) and then filtered and evaporated to drying. For the spectrophotometric determination, 5 g/l of the various extracts from each sample of rice leaves were used.

- **Estimation of total phenolic content:** The total phenolic contents in the leaves were determined using gallic acid as a standard phenolic compound as described by Yizhong *et al.* (2004) and Macheix *et al.* (2005). The solution was prepared in hemolysis tubes with blank reagent, standard (gallic acid) and the samples that were to be determined. Some 2 ml of chromogen prepared with Folin-Ciocalteu was added to 20  $\mu$ l of distilled water to form the reagent or the blank. Moreover, 2 ml of chromogen was added to 20  $\mu$ l of the reagent to form the standard (gallic acid at 3 g/l). Finally, 2 ml of chromogen was added to 20  $\mu$ l of each sample (methanolic extract leaves at 0.005 g/ml). Each solution was agitated using a vortex and allowed to rest for 1 minute. Then 1 ml buffer solution of sodium carbonate was added to each sample solution and agitated once again. The absorbance of sample solution and the standard are measured at 620 nm and

compared with that of the distilled water from the blank reagent.

- **Determination of total tannins content :** The method used was based on the complex formation of protein-tannins content described by Hagerman and Butler (1978). However, some modifications were made following Soulama *et al.* (2014). The first buffer solution A was prepared by dissolving 9.8 g of NaCl in 800 ml of distilled water. Some 11.5 ml of glacial acetic acid was titrated with one liter of NaOH solution to adjust the pH to 4.9. A buffer B was prepared and composed of a mixed solution [(1% Sodium Dodecyl Sulphate (SDS) and triethanolamine 5% (TEA) v/v] and adjusted to pH 9.4 using  $H_3O^+$  and  $Cl^-$ ). To measure the absorbance due to the presence of tannins in the precipitate, spectrophotometry with 510 nm was used in a blank and the extract replaced by methanol.

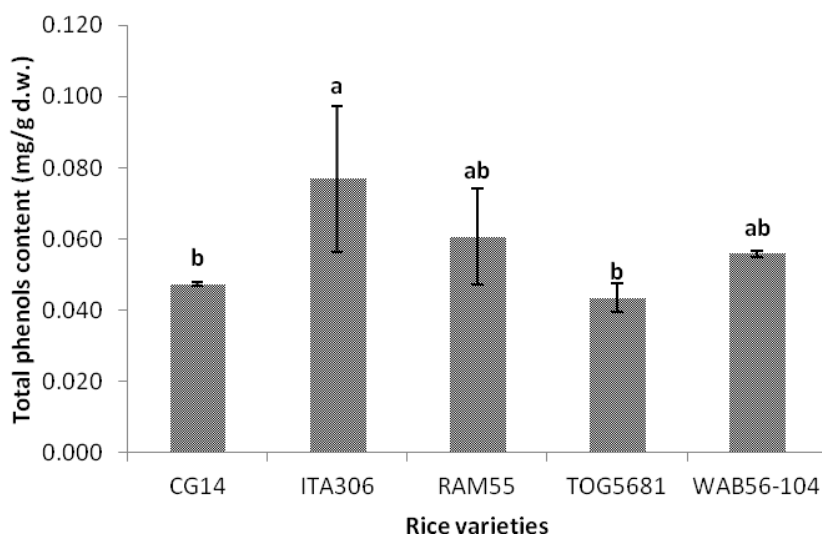
- **Determination of total anthocyanins content:** The content of anthocyanins in the examined plant extracts was determined spectrophotometrically as described in Lee *et al.* (2005). Required were buffer solutions of KCl (pH=1) and sodium acetate (pH=4.5); standard (cyanidin - 3 - glucoside) and the sample solutions to be determined. Potassium chloride; (0.25 M) buffer was added to 980 ml of distilled water to make approximately 1.86g of KCl in a beaker. The pH of the mixture was measured with a pH-meter and adjusted to pH= 1 ( $\pm 0.05$ ) with 6.3 ml of ( $H_3O^+$ ,  $Cl^-$ ). The solution was poured into a volumetric flask of 1 liter, and topped with additional distilled water. Sodium acetate pH=4.5 (0.4M) buffer was added in a beaker: 54.43 g of  $CH_3CO_2Na$ ,  $3H_2O$ , approximately 960 ml of distilled water. The absorbance of each dilution was respectively read at 520 and 700 nm, and compared to a blank made up of distilled water (2.10 ml).

- **Determination of total flavonoids content:** The flavonoids content was determined spectrophotometrically as described in Hariri *et al.* (1991). A standard stock solution of quercetin (0.1 mg/ml) was prepared from successive dilutions (0.001 to 0.03 mg/ml); and a solution of the sample to be measured was prepared from 80% MeOH (Methyl alcohol or Methanol). 100  $\mu$ l of the 1% NEU (2-aminoethyl diphenyl borinate) reagent was added to 2 ml of the extract solution. After vortexing, the absorbance was measured at 404 nm at the colorimeter regarding to the blank MeOH 80% (2.10 ml). Each experiment was repeated thrice and calibration was carried out using quercetin as a standard.

**Statistical analysis:** The relationship between the spectrophotometric absorbance readings and



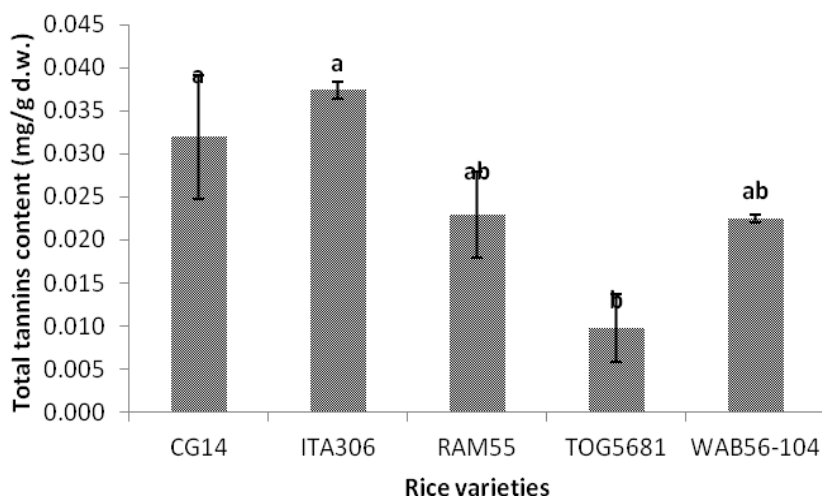
ITA306 (0.077 mg/g d.w.) and the lowest content in TOG5681 (0.043 mg/g d.w.).



**Figure 1:** Total phenols content in leaves of five rice varieties (n= 3; vertical bars are standard errors). Values with same letter are not significantly different at  $p<0.05$ .

- **Total tannins contents:** The contents of total tannins in the respective rice varieties were shown in figure 2. It is ranges from 0.010 to 0.037 mg/g d.w. and showed significant differences between rice varieties. Their respective values were 0.032; 0.037; 0.023; 0.010 and 0.022 mg/g d.w. for CG14, ITA306, RAM55,

TOG5681 and WAB56-104. Thus, the highest content in total tannins was obtained in rice variety ITA306 (0.037 mg/g d.w.); whereas the lowest content in total phenols was obtained in rice variety TOG5681 (0.010 mg/g d.w.).



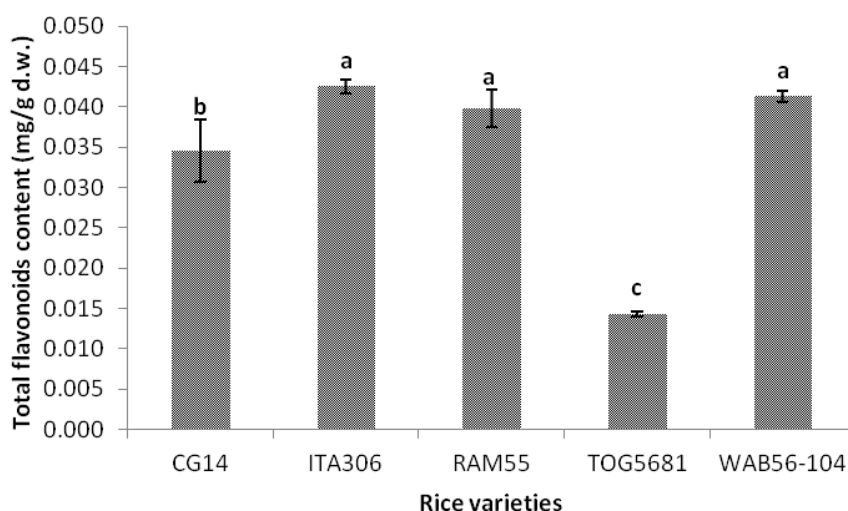
**Figure 2:** Total tannins content in leaves of five rice varieties (n= 3; vertical bars are standard errors). Values with same letter are not significantly different at  $p<0.05$ .

- **Total flavonoids contents :** The content of total flavonoids in each of the five rice varieties was

shown in figure 3. It ranges from 0.014 to 0.043 mg/g d.w. with significant differences between varieties. Their

respective values were 0.035; 0.043; 0.040; 0.014 and 0.041 mg/g d.w. for CG14, ITA306, RAM55, TOG5681 and WAB56-104. Variety ITA306 and WAB56-104

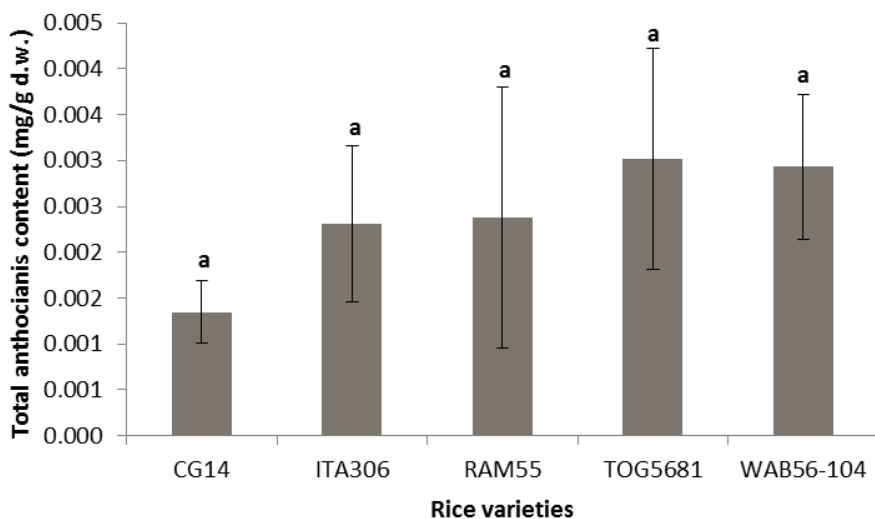
(0.041 mg/g d.w.) recorded the highest content in total flavonoids followed by RAM55 and lastly TOG5681 (0.014 mg/g d.w.).



**Figure 3:** Total flavonoids content in leaves of five rice varieties (n= 3; vertical bars are standard errors). Values with same letter are not significantly different at  $p < 0.05$ .

- **Total anthocyanins contents:** The content of total anthocyanins in each of the five rice varieties was shown in figure 4. It ranges from 0.001 to 0.003 mg/g d.w. with no significant difference between rice

varieties.. Their respective values were 0.001; 0.002; 0.002; 0.003 and 0.003 mg/g d.w. for CG14, ITA306, RAM55, TOG5681 and WAB56-104.



**Figure 4:** Total anthocyanins content in leaves of five rice varieties (n= 3 replications; vertical bars are standard errors). Values with same letter are not significantly different at  $p < 0.05$ .

## DISCUSSION

This study results revealed the presence of various classes of molecules such as phenolic compounds (tannins and flavonoids), mucilages, heterosides and reducing compounds in all the five rice varieties. Indeed, plants contain several classes of chemical compounds (Debella *et al.*, 2008) which have multiple functions mostly in reducing the digestibility of plant by insect (e.g. phenolic compounds and reducing compounds). They protect plants against herbivores like bites and insects pests (Malaka, 1972; Yousif & Satti, 2008). Terpenoids (essential oils) were present only in variety WAB56-104. Terpenoids are known to be involved in animal attraction indicating a likelihood of an existing relationship between this rice variety and herbivores. Reducing sugars were highest in CG14 among the others. Saponosids (heterosides) were also detected only in CG14. These compounds have varied biological and pharmacological principles. They are known for their antimicrobial and insecticidal activity (Djahra, 2014). Variety CG14 is a relevant of the African traditional rice species (*Oryza glaberrima*) known for its ability to tolerate abiotic and biotic stresses that are endemic in African continent (Wang *et al.*, 2014). In particular, this variety is known to be resistant to *Diopsis apicalis*, one of the devastating insect of this crop (Togola *et al.*, 2011). The presence of these specific compounds in the leaves of CG14 is suspected to be involved in the resistance of this variety to pests especially to *Diopsis apicalis*. Triterpenoids are widely present only in the variety WAB56-104. They include groups of biologically important substances that comprise sterol and steroids hormones. The basic structure is the common sterane molecule (Djahra, 2014) which has insecticide and repulsive actions in *Tribolium castaneum* (Khan *et al.*, 2014). The variety WAB56-104 was reported to be susceptible to *Diopsis apicalis* (Togola *et al.*, 2011). A high level of mucilages was observed in varieties ITA306, WAB56-104, TOG5681 and RAM55 but were absent from CG14. In higher plants, mucilages are mainly in the sap but their utility for plants was not well known. Our results revealed a significant difference among the five rice varieties in their total phenol content. These findings confirm previous results obtained on seeds of seven apple varieties (Ying *et al.*, 2016). The total phenols are very essential to protect crops against pests (Sharma *et al.*, 2009; Usha Rani & Jyothsna, 2010; War *et al.*, 2011). Their different roles in the protection of plants are widely studied and they can be used as solution against pests (Usha Rani & Jyothsna, 2010; War *et al.*, 2012). Wójcicka (2010) reported that the concentration

of total phenols is very high in the flag leaves of wheat and that they are very active in the control of some wheat aphids. They act on the behavior, physiology and metabolism of pest insects and hence reduce the pest population on plants (Bennett and Wallsgrave, 1994; Leszczyński, 2001). Thus, the fact that the content of these compounds was higher in variety ITA306, RAM55 and WAB56-104 suggest that these varieties should be more resistant to pests attack. Conversely, TOG5681, with the lowest content in total phenols, is supposed to be more relatively sensitive to pests.

Tannins are a complex group of polyphenolic compounds found in a wide range of plant species commonly consumed by ruminants (Sakshi & Srishti, 2015). These molecules are known characteristics of small group of families of Anarcadiaceae, Fagaceae, Ericaceae, Geraniaceae and Aceraceae (Biaye, 2002). Tannins play relevant roles in plant resistance against herbivorous insects (Moctezuma *et al.*, 2014). Tannins are disincentives that reduce the digestibility of food in the gut of insects. They exhibit the ability to disrupt or block the synthesis of molecules including proteins (Robbins *et al.*, 1987; Haslam, 2007). This role of tannins is in contradiction with observations that appear to be beneficial to plant-insect interactions (Ayles *et al.*, 1997; Heil *et al.*, 2002). These molecules could possibly influence the preference of host plants by the female insect for the survival of her offspring. For example, tannins may sometimes act as toxins for herbivorous insects but as facilitators of digestion (Rey *et al.*, 1999; Barbehenn *et al.*, 2008). Our results revealed a significant difference among the five rice varieties in their total tannins content. Thus, the fact that the contents of these compounds is higher in varieties ITA306 and CG14 lead to the supposition that they may be more resistant to herbivorous insects and TOG5681, with the lowest content in total phenols be relatively sensitive to herbivorous insects. Flavonoids are a complex group of polyphenolic compounds found in a wide range of plant species. They are known to have very complex roles in plant insect interactions (Harborne, 2001; Simmonds, 2003); and may have a positive or negative role in the life of the herbivorous larvae depending on the species of pests and the type of chemical composition (Harborne & Grayer, 1994; Renwick *et al.*, 2001; Simmonds, 2003). For example, quercetin and its glycosides reduced the growth of some moth larvae *Lymantria dispar* L. line in different species of pine (Beninger & Abu Zaid, 1997). Flavonoid aglycones in the leaves of birch had a defensive role against the

moth *Epirrita autumnata* in autumn (Lahtinen et al., 2004). In a previous study, Rezazadeh et al. (2007) reported that total flavonoids content in leaves of *Ginkgo biloba* L. cultivated in Iran varied significantly during different months with a highest concentration obtained in younger tree leaves. The relationship between the highest total flavonoids content and resistance of pests must be studied in rice varieties ITA306, WAB56-104 and RAM55. In addition, relationship between the lowest total flavonoids content and the relative sensitivity of pests in rice variety TOG5681 should be studied. The significant variation among rice varieties in their contents of anthocyanins confirm previous results obtained on 190 samples of black raspberry (*Rubus occidentalis* L.) over two growing seasons (Dossett et al., 2010). Those researchers found significant difference between black raspberry lines for their content in anthocyanins and they showed high heritability for its content. Anthocyanins are visible products of flavonoid biosynthesis, and contribute to colouring of flower and fruits and for attraction of pollinators. Their roles in plant defense against herbivores and pathogens are not known (Onyilagha et al., 2004). All the varieties studied were similar in concentrations of anthocyanins. This molecule does not appear to play an important role in

protecting plants against diopsids. Varieties with high anthocyanin content such as WAB56-104 were highly susceptible to insect damage. This result confirms the finding that purple and blue anthocyanins are particularly attractive to many insects, such as bees, flies, and other pollinators; even to the hummingbirds (Glover & Martin, 2012). Other previous researches revealed that anthocyanins are integrated into the plant's strategies for survival by attracting or repelling pollinators and seed dispersers; they serving protective roles as shields against abiotic stresses like UV (ultraviolet)-B radiation, visible light, temperature variation, etc., and active defensive roles against pathogens, insects and herbivores (Andersen & Jordheim, 2010). Researches also mentioned that anthocyanins are known to act as feeding deterrents and bind with nitrogen, which may explain why beetles avoided feeding on ninebark in preference tests (Tenczar & Krischik, 2007). It is important to note that CG14, RAM55 and TOG5681 belong to *Oryza glaberrima* (African traditional rice species) while WAB56-104 and ITA306 belong to *O. sativa*. Thus, the significant differences among the five rice varieties studied in their total phenolic, flavonoids and tannins compounds, could be inherited from diversity.

## CONCLUSIONS

The present study revealed that rice leaves contain varying levels of secondary metabolites mainly in the groups of phenolic (tannins and flavonoids), mucilages, heterosides and reducing compounds. Alkaloids were absent in all the five varieties. There is a high variability of secondary metabolites profile and contents in rice varieties. Varieties WAB56-104, RAM55 and ITA306 contained more phenols, more flavonoids and more tannins than the two other tested varieties. These

results confirm that there is a variability in both profile and concentration of secondary metabolites in rice plants and suggest that the presence and/or the content of a specific metabolite could play a key role in rice insects' resistance. The present findings could be used to establish the relationship between secondary metabolites profile and contents for further use for managing rice insect pests.

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