Metabolic profiling of various mango cultivars to determine biomarkers for the protection against *Fusarium* and *Procontarinia* infestations

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ABSTRACT
Mango cultivars display variable susceptibility to *Procontarinia* and *Fusarium* infestations. High incidence of gall fly attack has been linked to attraction of the insect by volatiles emitted from flush leaves. However, some cultivars demonstrate the ability to inhibit the maturation of the insect, resulting in the development of pseudogalls, rather than true galls.

*Fusarium* infections lead to malformation of mango flowers and young shoots. The only recourse at this stage is to remove branches up to 50 cm from the point of infection. Some mango cultivars, ‘Sunshine’ and ‘Roza’, are tolerant to blossom malformation.

Secondary metabolites within the plant may be responsible for the protection rendered against *Procontarinia* and *Fusarium* infestations. Chemical profiles of mature leaves of cultivars with varying degrees of resistance to gall formation and *Fusarium* infection were obtained by HPLC analysis. The data obtained was used to construct chemometric models that identified biomarkers responsible for protection against *Procontarinia*, as well as *Fusarium*.

From the biomarker identification it seems as if the compounds responsible for resistance against *Procontarinia*, may be responsible for susceptibility to *Fusarium* infestation. The identified components must be applied to infested mango plant structures to determine the protection offered.

ABSTRAK
Mangokultivars toon wisselende vatbaarheid vir *Procontarinia* en *Fusarium* besmetting. Hoë voorkoms van galvlieg-aanvalle was gekoppel aan die aantrekking van die insekte deur vlugtige organiese stowwe wat deur baie jong blare uitgeskei word. Sommige kultivars het egter die vermoe om die ontwikkeling van die insek binne die gal te staak, wat tot die vorming van pseudogalle in plaas van ware galle lei.

*Fusarium*-besmetting veroorsaak misvorming van bloeisels en jong takkies. Huidig is die enigste oplossing die uitbreek van alle takke tot op 50 cm vanaf die punt van infestasie. Sommige mangokultivars, ‘Sunshine’ en ‘Roza’, is meer bestand teen bloeiselmisvorming.

Sekondêre metaboliëte binne die plant mag verantwoordelik wees vir die beskerming wat teen *Procontarinia*-en *Fusarium*-infestasies gebied word. Chemiese profiele van volwasse blare van kultivars met verskillende grade van vatbaarheid teen galvorming en bloeiselmisvorming is verkry deur HPLC analyses. Hierdie data is gebruik om chemometriese modelle op te stel wat biomerkers, verantwoordelik vir die beskerming teen *Procontarinia* asook *Fusarium*, geïdentifiseer het.

Dit wil voorkom asof die biomerkers verantwoordelik vir beskerming teen *Procontarinia* gepaard gaan met verhoogde vatbaarheid vir *Fusarium*-infestasie. Die geïdentifiseerde verbinding moet tot besmette mangokultivars toegedien word om sodoende die mate van beskerming te bepaal.
INTRODUCTION
High incidence of gall fly attack has been linked to attraction of the insects by volatile organic compounds emitted from flush leaves (Augustyn et al., 2010a). However, some cultivars demonstrate the ability to inhibit the maturation of the insect, resulting in the development of pseudogalls, instead of true galls. Secondary metabolites produced by the leaves may be responsible for this observation.

In a previous study it was found that mangiferin, a secondary metabolite produced by various mango plant parts, may play a role in pseudogall formation (Augustyn et al., 2010b). ‘Keitt’, a pseudogall-forming cultivar as well as ‘Sensation’, a resistant cultivar, have higher levels of mangiferin than the true gall forming cultivar, ‘Heidi’. Mangiferin has an arresting effect on proliferation of tumours and malformed mammalian cells (Masibo & He, 2008) and has been shown on proliferation of tumours and malformed mammalian cells (Masibo & He, 2008) and has been shown to stimulate the release of defence-related enzymes (Singh, 2006). These actions might be responsible for the resistance afforded against Procontarinia. Extracts of bark or leaves displayed the same activities as the pure compound. Mangiferin was also shown to cause lysis of hyphal cells, to retard mycelial growth of Fusarium and inhibit the production of fusaric acid, a metabolic product produced by the fungus (Ghosal et al., 1977).

Mango malformation is caused by Fusarium infestations and is the most significant floral disease of mango (Kvas et al., 2008). This disease limits fruit production and subsequently results in substantial economic losses. Fungal growth can be inhibited in vitro by plant secondary metabolites that are present in essential oils and plant extracts (Combrinck, Regnier & Kamatou, 2011; Linde et al., 2010).

Metabolomics is the simultaneous assessment of the entire set of metabolites produced by biological matrices. Metabolic profiles of wheat cultivars, to determine biomarkers for resistance and susceptibility to Fusarium head blight, revealed that specific metabolites are related to cultivar resistance (Hamzehzarghani et al., 2008). In this study, biomarkers associated with resistance to gall fly infestation and Fusarium infection, were identified using cultivars with varying susceptibilities.

MATERIALS AND METHODS
Mature leaves of the selected cultivars (‘Heidi’, ‘Tommy Atkins’, ‘Keitt’, ‘Kent’, ‘Roza’ and ‘Sunshine’) were obtained from orchards in Hoedspruit, Mpumulanga. Leaves were dried in an oven (Protea Laboratory Instruments, Labotec, Johannesburg, South Africa) at 40°C until constant mass. Dried leaves were ground using a Genevac (EZ-2 personal evaporator, United Scientific, South Africa) before adjusting the volumes to 5 ml with distilled water. Chlorophyll was removed from each extract by adding 5.0 ml chloroform to the tube, vortexing for 1 min and centrifuging at 8 000 rpm for 5 min. The chloroform layer was discarded. After two chloroform extractions, the final volume of the aqueous phase was adjusted to 5 ml with 20% aqueous methanol.

Samples were diluted 1:5 with 20% aqueous methanol before high performance liquid chromatography (HPLC) analysis. Extracted phenolic compounds (20 μl) were separated on an Inertsil C-18 reversed phase column (250 mm x 4.6 mm i.d. x 5 μm particle size), using two solvents: Solvent A (distilled water acidified with orthophosphoric acid (pH 3.5 ± 0.02)) and Solvent B (acetonitrile). The gradient program was as follows: initially Solvent A:B (95:5) ramped to 75:25 in 25 min, ramped in 10 min to 20:80, held for 5 min and back to A:B (95:5) in 5 min. Compounds were detected with a fixed wavelength UV detector at 280 nm.

Multivariate analysis of the HPLC data of all peak areas was used to construct various chemometric models to determine the relationships between the datasets. SIMCA-P+ (13.0) software (Umetrics, Sweden) was used following univariate (UV) scaling of the data. Analysis by orthogonal partial least squares (O-PLS) was done to establish a model enabling the prediction of susceptibility or resistance of mango cultivars to gall fly attack. Scatter, loadings and S-plots were set up to visualise results and predict possible biomarkers for both pests.

RESULTS AND DISCUSSION
Identification of biomarkers for Procontarinia
Comparison of the HPLC profiles (Fig. 1) of leaf extracts from true gall-forming ‘Heidi’ and ‘Tommy Atkins’, to a pseudogall-forming cultivar, ‘Keitt,’ indicated differences between the two types of cultivars. It is evident that the pseudogall-forming cultivar ‘Keitt’, produces the highest levels of the compound that elutes at 18 min and a peak at 17 min with the second highest intensity. The other two cultivars, ‘Heidi’ and ‘Tommy Atkins’, are characterised by a major compound eluting at 14 min, with the compounds at 17 and 18 min at lower levels.

In this study, qualitative and quantitative data obtained from HPLC analyses were statistically processed using O-PLS analysis; an approach frequently used in chemometric analysis (Eriksson et al., 2006). Orthogonal-PLS scores plot, constructed from the HPLC data of ‘Heidi’, ‘Tommy Atkins’ and ‘Keitt’, indicated clear clustering according to gall type, true or pseudogall forming (Fig. 2A). These results suggest that there are inherent chemical compositional differences between the two types of gall-forming cultivars. The high values for R²Y (0.86) and Q² (0.78) indicate good separation based on class differences and the high predictivity of the model. The closer these values are to 1.0 the better the model. The loadings column plot (Figure 2B) indicates the contribution of each compound (given as retention time) to the formation of either true galls or pseudogalls. All components
with a positive loading contribute to pseudogall formation, while those with negative loadings contribute to true gall formation. The jack-knife confidence limits reflect the uncertainty associated with the contribution made by a specific component and is directly correlated to reliability. Compounds with jack-knife confidence limits that cross the zero line reflect a large degree of uncertainty. Metabolites with confidence limits that do not cross the zero mark are 95% statistically safe (Eriksson et al., 2006). Components that contribute the most to pseudogall formation with reliable confidence limits are those with retention times: 4.9, 14.6 (iriflophenone-3-C-β-D-glucoside) and 18.2 min (iriflophenone-3-C-(2-o-p-hydroxybenzoyl)-β-D-glucoside). In a previous study it was found that mangiferin may play a role in pseudogall formation. The metabolite identified in this study, iriflophenone-3-C-(2-o-p-hydroxybenzoyl)-β-D-glucoside, is very close in structure to that of mangiferin. The abilities of these compounds, identified from their mass spectra, to prevent the development of gall fly larvae inside the leaves, must be assessed.

**Identification of biomarkers for *Fusarium***

Differences in metabolite levels can be observed from the HPLC profiles (Fig. 3) of leaf extracts from cultivars with varying susceptibility, ‘Keitt’ (highly susceptible), ‘Roza’ and ‘Susnshine’ (tolerant) to *Fusarium* infestation. These differences may contribute to the differences in susceptibility. It can be seen that ‘Keitt’, the highly susceptible cultivar, produces the highest levels of the component eluting at retention time 18 min and the component at 17 min as the second major compound. ‘Roza’ contains high levels of a compound with a retention time of 11 min, while that at retention time 18 min is present at much lower levels. ‘Sunshine’ is characterised by a simpler profile containing only two major compounds present at 14 and 17 min.

OPLS scores plots of *Fusarium* susceptible and resistant cultivars (Fig. 4) display clear clustering according to class. In Fig. 4A, the separation between mid-susceptible, ‘Heidi’ and ‘Tommy Atkins’, to resistant, ‘Roza’ and ‘Sunshine’ is illustrated, while Figure 4B is a plot indicating the separation between highly susceptible, ‘Keitt’ and ‘Kent’, and resistant, ‘Roza’ and ‘Sunshine’. The high $R^2_Y$ values, 0.908 and 0.804, respectively, and low $R^2_X$ values indicate that the separation between classes is much bigger than the variation between the components within a class.

**Figure 1.** HPLC chromatograms of microwave extracts of mature leaves of ‘Keitt’, ‘Heidi’ and ‘Tommy Atkins’.
The high $Q^2$ values obtained for both models (0.876 and 0.79 respectively) indicate the high predictability of the models.

The loadings column plots with jack-knife confidence limits (Fig. 5) reflect the contribution of each compound (given as retention time) to either resistance or susceptibility. Fig. 5A displays the loadings column plots of mid-susceptible cultivars ‘Heidi’ and ‘Tommy Atkins’ to the resistant cultivars, ‘Roza’ and ‘Sunshine’. Figure 5B is the loadings plot of the highly

**Figure 2A.** OPLS scores plot constructed from HPLC data comparing true gall formation, ‘Heidi’ and ‘Tommy Atkins’, to pseudogall formation, ‘Keitt’. B: Loadings column plots with confident limits for compounds that contribute to the differences between true gall and pseudogall forming cultivars.

**Figure 3.** HPLC chromatograms of microwave extracts of mature leaves of ‘Keitt’, ‘Roza’ and ‘Sunshine’. 
susceptible ‘Keitt’ and ‘Kent’, compared to the resistant cultivars. All components with a positive loading contribute to resistance and vice versa, to susceptibility to *Fusarium* infestation. From this plot, the component that contributes the most to resistance is the one with retention time 11 min (maculrin-3-C-β-D-glucoside). The components with retention times 17.3 (mangiferin), and 29.2 min (kaempferol) contribute to resistance, but to a lesser extent. The components that contribute the most to susceptibility are compounds with retention times at 4.9 (unidentified), 18.2 (iriflophenone-3-C-(2-o-p-hydroxybenzoyl)-β-D-glucoside) and 25.5 min (quercetin). The compounds have only been tentatively identified and must be verified with authentic standards.

From the data obtained it seems as if the components responsible for resistance against *Procontarinia* (compounds eluting at retention times 4.9 and 18.2 min), might be linked to susceptibility to *Fusarium* infestation. The application of these compounds as plant extracts or pure compounds will therefore have to be timed accurately to ensure maximum protection. Orchard sprays of the identified compounds must be applied in October and March to protect the trees against *Procontarinia*, while those against *Fusarium* must be applied before and during bud formation up until fruit set at two week intervals. The data obtained will permit new cultivars to be screened for the presence of the identified metabolites before a decision on cultivation is made.

**CONCLUSION**

Chemometric analysis of data obtained point out significant differences between metabolites produced by the leaves of cultivars forming true and pseudogalls in response to *Procontarinia* exposure. Biomarkers for pseudogall formation have been identified and should be tested as foliar sprays before and after infestation of flush leaves by the gall fly. Analogously, biomarkers for susceptibility and resistance to *Fusarium* infestation have been identified and must be tested. OPLS analysis of the metabolomics data is an unbiased method to identify compounds that play a role in resistance and susceptibility to both these orchard pests.

In future, data must be obtained from additional cultivars to ensure robust prediction models that can be used to determine susceptibility of new cultivars.
to these orchard pests before large scale cultivation. Electron microscopy is a useful tool that can be employed to determine the extent to which infestation has been halted by application of biomarker compounds.

REFERENCES