Effect of Rooibos and Honeybush Tea Extracts Against *Botrytis cinerea*

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Green tea extracts from the indigenous South African rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* species) plants were evaluated as potential antifungal agents against the plant pathogen *Botrytis cinerea*. When applied at 10 mg/ml, the tea extracts stimulated biomass production in *B. cinerea* by more than 3-fold after 24 hrs. This induction could not be linked directly to the presence of selected micro- and macronutrients or antioxidants in the extracts, suggesting a complex set of yet unidentified factors that may act synergistically to enhance cell growth. However, when applied at 100 mg/ml, the *A. linearis* and *C. genistoides* extracts reduced spore germination of *B. cinerea* by 33.3% and 16.7%, respectively. This suggests that the tea extracts contain active compounds that should be further investigated for their potential as natural anti-fungal agents.

Botrytis cinerea is the principle microorganism responsible for 'grey mould' on grapes and other fruits and vegetables that could result in severe crop damage (Ribéreau-Gayon *et al*., 2006). The fungus is characterised by abundant hyaline conidia (asexual spores) or highly resistant sclerotia that enables it to survive winter or other unfavourable periods (Mendgen & Hahn, 2002). Grey mould is commonly associated with humid conditions and temperatures of 10-25°C, with the presence of water on the surface of the grape berry and a temperature of 18°C being ideal for germination and mycelial growth (Ribéreau-Gayon *et al.*, 2006). The fungus is a necrotrophic pathogen that actively kills plant cells and subsequently lives on dead tissue. Infection seldom occurs before *véraison* and is dependent on some kind of damage to the berry skin to provide a point of entry, e.g. physical damage by hail or parasites, or dislodged berries that are imprisoned inside the grape cluster. In addition to the production of antifungal inhibitors, the thick cuticle of the grape berry provides mechanical resistance against *B. cinerea* infection (Ribéreau-Gayon *et al.*, 2006). After *véraison*, the grape's resistance to *B. cinerea* are weakened due to various chemical and physical changes, with the presence of microperforations of the cuticle and stomatic fissures providing a point of entry for growing hyphae.

Infection by *B. cinerea* in grapes are usually controlled by canopy management, preharvest spraying with fungicides and postharvest sulphur dioxide fumigation (Romanazzi *et al*., 2007). However, sulphur dioxide causes bleaching of the berries and its residues can be allergenic, while fungicides have little effect if the prevailing weather conditions are inducive to infection (Reino *et al*., 2004). In the pursuit to develop alternative and safe antimicrobial preservatives, the antimicrobial properties of various plant and herb extracts and essential oils have been investigated (Feng & Zheng, 2007; Lee *et al.,* 2007; Tzortzakis & Economakis, 2007; Xu *et al.,* 2007). These natural compounds satisfy consumer demand, since the use of certain hormones and synthetic chemicals have been restricted due to their possible carcinogenicity and toxicity, long degradation period, environmental pollution and human side effects (Feng & Zheng, 2007).

Extracts of *A. linearis* and *Cyclopia* spp. are known for their anti-oxidant, anti-mutagenic and anti-carcinogenic activities (Von Gadow *et al*., 1997; Hubbe & Joubert, 2000; Joubert *et al*., 2003b; Marnewick *et al.*, 2000, 2005; Van der Merwe *et al*., 2006). Studies on the phenolic content of *C. intermedia* E. Mey and *C. subternata* have revealed the presence of phenolic metabolites thought to have significant pharmacological properties (De Nysschen *et al.*, 1996; Kamara *et al*., 2003; 2004). The major phenolic compounds in honeybush plant material are the xanthone, mangiferin (a C-glycoside), and the flavanone O-glycoside, hesperidin (Joubert *et al.*, 2003a; Van der Merwe *et al*., 2006).

In our search for alternative eco-friendly control systems for *B. cinerea*, consideration was given to the possible inhibition of *B. cinerea* with extracts prepared from rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* species), two indigenous South African plants rich in antioxidants that are used for the production of herbal teas as well as extracts for neutraceutical, health and beauty products. The inhibition of rooibos extracts against *E. coli* and other selected bacterial and yeast strains was previously reported by Schepers (2001). To our knowledge, the efficacy of extracts from *A. linearis* and *Cyclopia* spp. as anti-fungal agents against *B. cinerea* has not been reported.

MATERIALS AND METHODS

Plant material and reagents

Green (unfermented) tea was used as plant material due to the general higher antioxidant content of green tea relative to that of fermented tea (Joubert *et al.*, 2003b). Dried extracts prepared from rooibos (*A. linearis*) and honeybush (*Cyclopia subternata*) tea were supplied by Raps GmbH & Co. (Germany), while the *Cyclopia genistoides* tea extract was supplied by the Post-Harvest & Wine Technology Division of ARC Infruitec-Nietvoorbij (Stellenbosch, South Africa). The total antioxidant activity (TAA) of

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the extracts was determined using the $ABTS^{\bullet+}$ scavenging assay (Re *et al*., 1999).

Stock solutions of green tea extracts were freshly prepared by dissolving the extract powder in 70% ethanol at a concentration of 1 g/mL with further dilutions as required. All chemicals were of standard or analytical grade and obtained from BDH Chemicals Ltd (Poole, England); Fluka AG (Buchs, Switzerland); Merck (Darmstadt, Germany) or Sigma Chemical Co. (St. Louis, USA).

Strains and culture conditions

Escherichia coli DH5α was cultured overnight at 37ºC in Luria Bertani (LB) medium (Hunt *et al*., 2005) and *B. cinerea* strain STEU 6253 (Dept. Plant Pathology, Stellenbosch University) in Potato Dextrose (PD) medium at 25ºC for 7 days (Rauha *et al*., 2000). All cultures were cultivated under constant agitation (100 rpm). Fungal spores were harvested in 100 mL 0.85% sodium chloride containing 1% Triton X-100 (BDH Chemicals Ltd).

Effect of tea extracts on cell growth in liquid cultures

Overnight pre-cultures were used to inoculate triplicate sets of 50 mL LB medium (*E. coli*) or PD medium (*B. cinerea*) at a final concentration of 10^5 to 10^6 cells or spores/mL. Extracts from *A. linearis* and *Cyclopia* spp were added at 10 or 100 mg/mL, with the controls not receiving any extract. Bacterial and fungal cultures were kept at 37°C and 25ºC, respectively, protected from light to minimise oxidation of the tea extracts. For *E. coli*, duplicate samples were taken at 0h, 6h, 12h, 24h and 48h and used to quantify viable bacterial cells by means of duplicate dilution plate counts. Percentage inhibition was expressed as $I = [(N_c-N_t)/N_c] \times 100$, where N_c and N_t represent the viable cell numbers for the control and treatments, respectively.

To determine biomass production by the fungal strains, triplicate samples were taken at 0h, 6h, 12h, 24h, 48h and 72h of which 1 mL was centrifuged at 13K for 15 minutes. The supernatant was decanted and the pellet dried overnight at 70ºC. Biomass was measured and expressed as mg dry weight/mL.

Effect of added nutrients and ascorbic acid on biomass production

PD medium with/without 10 mg/mL extracts from *A. linearis* and *C. subternata* were analysed for their micro- and macronutrient content (BemLab, Somerset West, South Africa). Based on the respective cation concentrations in 10 mg/mL *A. linearis* tea extract, an enriched PD medium was prepared with 48.558 mg/L KCl, 1.929 mg/L $CaCl_2$, 14.885 mg/L $MgSO_4.7H_2O$ and 0.139 mg/L $MnCl_2.4H_2O$. Spores from *B. cinerea* were inoculated in triplicate at a final concentration of 10^5 to 10^6 spores/mL into PD medium with or without added nutrients, as well as in PD medium plus 10 or 100 mg/mL ascorbic acid (Sigma Chemical Co., St. Louis, USA) to simulate the antioxidant content of the rooibos extract (TAA of 3204 mmole/g). The cultures were incubated at 25ºC and triplicate samples were taken at 0h, 6h, 12h, 24h, 48h and 72h, of which 1 mL was centrifuged at 13K for 15 minutes and dried overnight at 70ºC. The supernatant was decanted and the pellet dried overnight at 70ºC. Biomass was measured and expressed as mg/mL (dry weight).

Spore mortality

The toxicity of *A. linearis* and *C. genistoides* tea extracts to *B. cinerea* spores was evaluated as previously described by Karabulut *et al.* (2005), with minor modifications. In short, 10^4 spores/ml *B. cinerea* spores were mixed with the respective tea extracts (final

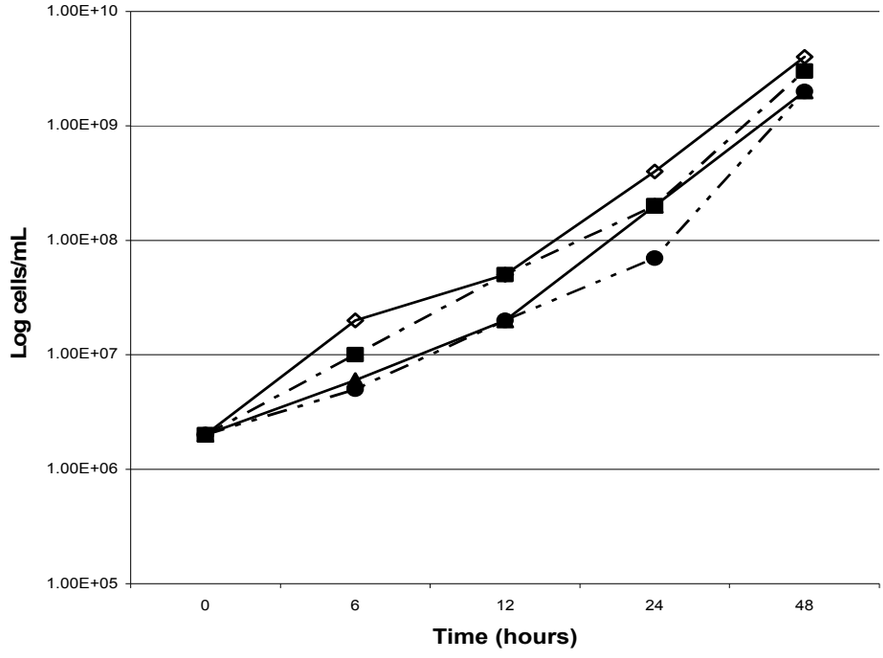

FIGURE 1

The effect of (■) 10 mg/mL *A. linearis*, (▲) *C. subternata* and (●) *C. genistoides* extracts on viable *E. coli* cell numbers, when compared to PD medium only (◇).

concentration of 100 mg/mL) at ambient temperature (22-24°C) in a final volume of 2 mL. After 1 min, triplicate samples of the spore suspensions were diluted 100-fold in 0.85% NaCl of which 100 µL were plated onto PD-agar plates. Spores that were exposed to 0.85% NaCl without tea extracts were used as a control in each set of experiments. The plates were incubated at 23°C for 3 days and the colonies enumerated. Data were expressed as the percentage of germinated spores relative to the control experiment.

RESULTS AND DISCUSSION

Effect on bacterial and fungal cell growth

The efficacy of the tea extracts used in the experiments was validated using *E. coli* as a benchmark. In liquid cultures, 10 mg/mL of the *A. linearis*, *C. genistoides* and *C. subternata* extracts inhibited growth of *E. coli* cells by 60%, 80 and 85% respectively after 6 hrs, when expressed relative to the control (Fig. 1). After 24 hrs, 25% and 50% inhibition by *A. linearis* and both *Cyclopia* spp. was observed, but the effect diminished thereafter. This is in line with Schepers (2001) who reported a 35% cell growth inhibition in *E. coli* after 12 hrs in the presence of a 5.0 g/L green rooibos tea extract.

A different phenomenon was observed for the *B. cinerea* strain: both the *A. linearis* and *C. subternata* extracts induced biomass production by *B. cinerea* more than 5-fold after 48 hrs when applied at 10 mg/mL (Fig. 2).

The apparent induction of biomass production by the *B. cinerea* strain in the presence of the tea extracts could be due to the presence of either growth factors (micro- and/or macronutrients) or antioxidants in the tea extract itself that could scavenge free radicals that could otherwise impair cell growth and viability. The antioxidant content of the different extracts as reflected by the TAA values (mmole Trolox equivalents/g powder), was found to be 3204 mmole/g for *A. linearis*, 1633 mmole/g for *C. subternata* and 1676 mmole/g for *C. genistoides*.

Effect of added nutrients and antioxidants on biomass production by *B. cinerea*

Chemical analysis of micro- and macronutrients in the growth media supplemented with tea extracts (Table 1) indicated that both the *A. linearis* and *C. subternata* extracts contributed significant levels of especially Potassium (K), Calcium (Ca), Magnesium (Mg) and Manganese (Mn) when added at 10 mg/mL to PD medium.

The PD medium containing 10 mg/mL *A. linearis* tea extract induced biomass production of *B. cinerea* by 2-3-fold at 48 hrs (Figure 3). Similar trends were observed with the *C. subternata* extract (data not shown). However, no significant induction in biomass production of *B. cinerea* was observed when cultured in an enriched Potato Dextrose (PD) medium containing K, Ca, Mg and Mn ions at similar concentrations identified in 10 mg/ml extract of *A. linearis*. It is thus clear that the induced biomass production in the presence of the tea extracts could not be ascribed to the mere presence of these nutrients in the tea extracts.

When applied at 100 mg/mL, the *A. lineraris* and *C. subternata* extracts induced biomass production by more than 4-fold and 6-fold after 48hrs and 72 hrs, relative to the control, with indications of a continued induction thereafter. However, only a 3-fold induction in biomass production of *B. cinerea* was observed in the presence of 100 mg/mL ascorbic acid at 48 hrs, with a steep decline thereafter. Given the TAA activity of 3783 mmole/g for ascorbic acid, 3204 mmole/g for *A. linearis* and 1633 mmole/g for *C. subternata*, it is clear that the antioxidant activity of the tea extract per se was not re-

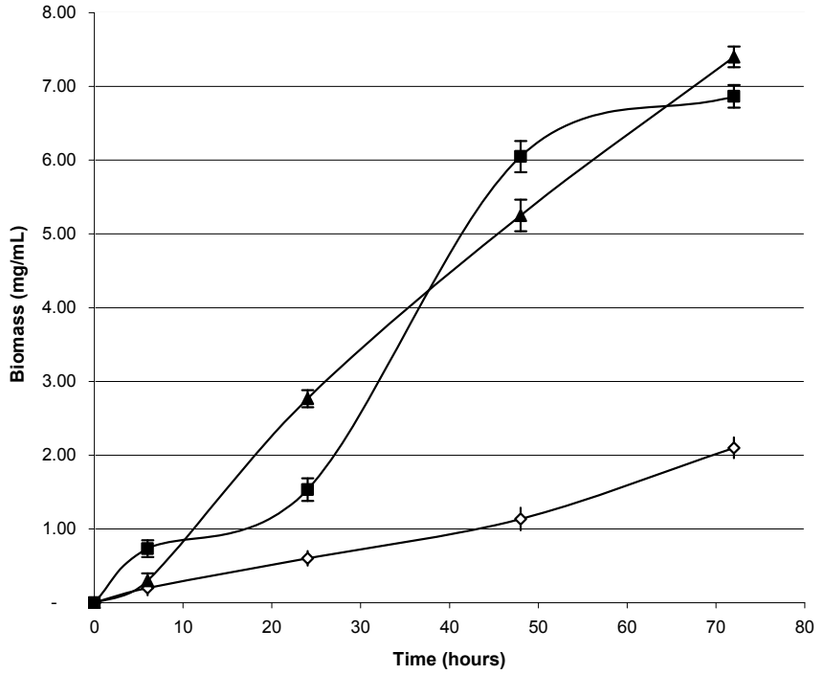

FIGURE 2

The effect of (■) 10 mg/mL *A. linearis*, (▲) *C. subternata* extracts on *B. cinerea* biomass production (dry weight), when compared to PD medium only (◇).

TABLE 1

Quantification of micronutrients (expressed as mg/L) contributed by Potato Dextrose medium and 10 mg/mL tea extracts. Cation concentrations in bold indicate those that were more than 50% higher in the extracts than in the control PD medium.

Media	N	P	K	Ca	Mg	Na	Mn	Fe	Cu	Zn	B
Potato Dextrose Medium (PD)	0.323	3.815	100.894	3.071	0.585	121.050	0.077	3.230	0.001	0.172	0.420
PD medium + 10 mg/mL *C. subternata* extract	0.429	4.397	**169.143**	3.607	**6.681**	136.541	**0.121**	3.548	**0.025**	0.187	0.455
PD medium + 10 mg/mL *A. linearis* extract	0.366	4.116	**149.452**	**5.000**	**15.470**	175.630	**0.216**	3.359	**0.004**	0.219	**0.633**

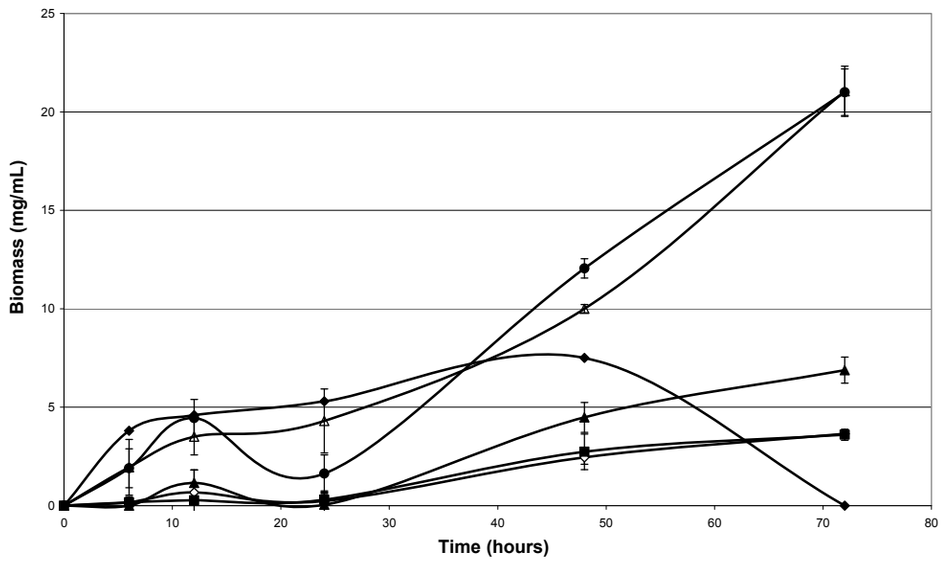

FIGURE 3

The effect of (■) added nutrients representing the major cations in 10 mg/mL *A. linearis*, as apposed to (▲) 10 mg/mL or (△) 100 mg/mL *A. linearis* extract, (●) 100 mg/mL *C. subternata* extract, and (◆) 100 mg/mL ascorbic acid on biomass formation by *B. cinerea* (dry weight), when compared to PD medium only (◇).

sponsible for the induced biomass production observed in the presence of the tea extracts. On the contrary, the PD medium containing ascorbic acid showed an initial increase in biomass similar to that of the tea extracts, followed by a steep decline in biomass towards 72 hrs, which suggest cell death as apposed to the further increase in biomass observed for the tea extracts.

Inhibition of spore germination in *B. cinerea*

Exposure of *B. cinerea* spores to *A. linearis* or *C. genistoides* tea extracts reduced spore germination by 36% (±0.7%) and 19% (±0.8%), respectively (Figure 4). The antioxidant content of the *A. linearis* extract (3204 nmole/g) was almost double that of the *C. genistoides* extract (1676 nmole/g), which may account for the different levels of inhibition of spore germination observed for the two tea extracts.

The antimicrobial effect of Chinese green tea has been directly linked to the presence of two bioactive flavanols namely, epicatechin gallate and epigallocatechin gallate (Si *et al.,* 2006). This suggests that the antifungal property of the tested tea extracts may be due to the presence of a specific flavanol or a combination of different flavanoids. Both rooibos and honeybush tea extracts are known to contain different phenolic compounds, including epicatechin gallate and epigallocatechin gallate (Kamara *et al.,* 2003; Kamara *et al.,* 2004; Rabe *et al.,* 1994).

The inhibition of *B. cinerea* spore germination observed for the *A. linearis* or *C. genistoides* tea extracts suggests the potential use of these extracts as antifungal agents. The use of chemicals such as sulfur dioxide and the emergence of fungicide-resistant strains within vineyard populations are all important factors driving the development of alternative antifungal agents. Currently, different essential oils, plant extracts and other substances are evaluated for their antifungal properties with regard to *B. cinerea*. These include grapefruit seed extract, chitosan, essential oils of thyme, sage and nutmeg, ethanol, potassium sorbate and carvacrol (Martínez-Romero *et al.,* 2007; Karabulut *et al.,* 2005; Feng and Zheng,

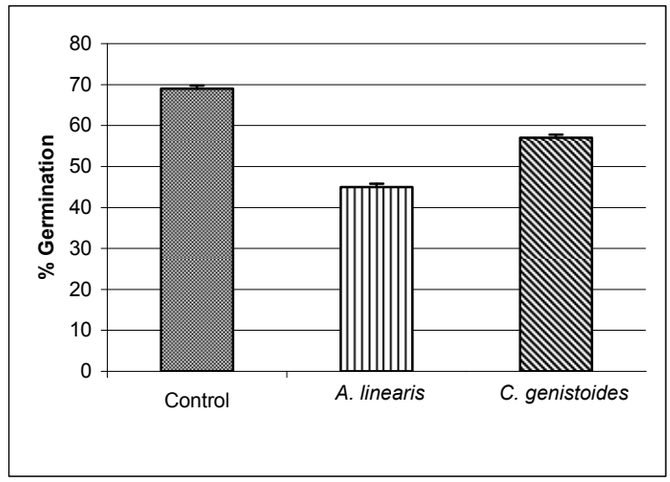

FIGURE 4

Germination of *B. cinerea* spores on PDA plates after exposure to 0.85% NaCl without tea extract (control), 100 mg/ml *A. linearis* extract and 100 mg/ml *C. genistoides* extract for 1 min.

2007; Xu *et al.,* 2007). It will be of great interest to isolate and identify these compounds and test their efficacy against a range of bacterial and fungal pathogens.

CONCLUSIONS

Results from this study showed that extracts from *A. linearis* and *Cyclopia* spp. have potential for inhibiting bacterial growth by 60%-85% when applied at 10 mg/mL. Both the rooibos and honeybush tea extracts have a bacteriostatic effect on *E. coli* cells, with the inhibitory effect diminishing after 48 hrs. If the anti-microbial efficacy of the tea extracts is linked to polyphenolic compounds in the extracts, it is likely that the anti-microbial efficacy of the respective tea extracts may diminish over time due to instability or oxidation of the active compound(s). Future studies can be done to isolate, identify and stabilise the anti-microbial components in the tea extracts with the intention to improve their efficacy.

In contrast to the observations with *E. coli*, the *B. cinerae* strain showed a significant increase in biomass production when cultured in the presence of extracts from *A. linearis* or *Cyclopia* spp. This may be ascribed to the presence of micro- and macronutrients, such as metal ions, in the tea extracts that are stimulating cellular growth. However, when some of the dominant micro- and macronutrients identified in the *A. linearis* extract were included in the growth medium, the effect was 2-fold less than in the presence of the tea extracts. The results also suggest that the mere presence of an antioxidant could not induce biomass production by *B. cinerea*. In fact, the *C. subternata* extract (TAA of 1633 mmole/g) was as effective as the *A. linearis* extract (TAA of 3204 mmole/g), with ascorbic acid (TAA of 3783 mmole/g) resulting in a two-fold less induction than the tea extracts after 48 hrs.

Tea extracts from *A. linearis* and *C. genistoides* decreased the spore viability of *B. cinerea* by 36% and 19%, respectively, suggesting that the extracts contain chemical compounds that could have potential as antifungal agents. However, these active compounds have to be isolated, identified and evaluated individually to ascertain their effect on *B. cinerea.* A better understanding of the active compounds will also assist us in elucidating the apparent conflicting effects on *B. cinerea*, i.e. the inhibition of spore germination and induction of biomass production observed in the presence of both *A. linearis* and *C. genistoides* extracts.

PATENT PENDING

Results presented here have been included in a patent entitled, "Plant extract having antimicrobial activity", filed with the South African patent office (ZA2007/08879).

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