A panel of bioluminescent whole-cell bacterial biosensors for the screening for new antibacterial substances from natural extracts

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Abstract

Whole-cell bacterial biosensors can be applied for the screening of antibacterial properties of extracts. We constructed a biosensor panel consisting of four different bacterial biosensor strains: *Escherichia*, *Staphylococcus*, *Acinetobacter* and *Pseudomonas* for expanded screening potential. The functionality of the panel was first evaluated with known antibacterial compounds: ethanol, naphthoquinones (juglone, lawsone, plumbagin) and a flavonoid (quercetin). Natural extracts comprise a vast source of potential new antibacterials for diverse functional purposes. To demonstrate the utilization of the panel for screening of a demanding sample material, round-leaved sundew *(Drosera rotundifolia*) extracts were used as an example. Differences between field- and laboratory originating sundew extracts could be detected. This demonstrates the efficiency of the developed biosensor panel in the rapid screening of the antibacterial properties of plant extracts.

Keywords: antibacterial, bioluminescence, biosensor cells, high-throughput screening, natural extracts, *Drosera rotundifolia*

1. Introduction

New antibacterial agents from sustainable and renewable natural sources are globally needed for variable purposes like preservatives in cosmetics, technochemicals, food or feed to replace synthetic preservatives. Furthermore, multi-drug resistant bacteria such as *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Acinetobacter baumannii* are becoming more serious global problem (Nair et al. 2016; De Bonis et al. 2016; Dhawan et al. 2017; Tenaillon et al. 2010), which emphasizes the need to find novel sources for potential pharmaceutical purposes. To combat these challenges, efficient methods are needed to screen nature-based sources for new antibacterial extracts and compounds.

Bacterial whole-cell biosensors can be used for screening antimicrobial effects. The bacterial biosensors can be constructed by genetic engineering to enhance the usability, for example by adding DNA elements to help recognize antibacterial activities. (Galluzzi & Karp 2006). One example of the DNA elements is a bacterial luciferase (*luxABCDE)* operon. However, using a single bacterial biosensor strain may give limited information about the antibacterial efficiency, as the activity might differ between bacterial species. Therefore, a biosensor panel consisting of multiple different bacterial biosensor strains can expand the screening potential.

Under normal conditions, the biosensor cells express *luxABCDE* and produce bioluminescence in a luciferase catalyzed reaction (Vesterlund et al. 2004). Bioluminescence is a useful detection method for the assays, as the light production is specific and detectable using very sensitive equipment. To estimate the sample

toxicity, the degree of inhibition of a 'normally on' activity is measured in "lights-off" biosensor assays (Belkin 2003). When the biosensor cells are cultivated with a cytotoxic compound, the gene transcription and protein translation become less active than without the cytotoxic compounds. As the toxicity increases, the inhibition escalates as well, lowering the intensity of the bioluminescent signal (Cui et al. 2018).

Natural extracts are a vast source of potential new antibacterials. A natural extract can comprise of up to 15 000 diverse metabolites, including both primary and secondary metabolites (Wolfender et al. 2015). Because of the complexity of natural extracts, testing the antimicrobial potential with just one bacterial species reveals only limited information about the full potential. Some previous studies have been made to determine antimicrobial effects of plant extracts on a panel of different bacteria (Rauha et al. 2000; Bacha et al. 2016). However, the methods used in these studies include disc diffusion and broth dilution assays, which are more labor- and time-intensive than using whole-cell bacterial biosensors.

Here, we developed a panel of bioluminescent "lights-off" bacteria and demonstrated its functionality with demanding sample matrices, sundew (*Drosera rotundifolia*) extracts. The panel contained four non-pathogenic bacterial strains: Gram negative species *E. coli*, *Acinetobacter baylyi*, *Pseudomonas putida* and Gram positive *S. aureus.*

2. Materials and methods

2.1. Chemicals

Labema, Finland supplied tryptone and yeast extract. NaCl, KH_2PO_4 were from Merck, USA. Kanamycin was purchased from Janssen, USA; erythromycin from TCI, Japan. K_2HPO_4 was from VWR International, USA. Glycerol, ampicillin sodium salt, juglone, lawsone, plumbagin, quercetin and Murashige-Skoog basal medium were purchased from Sigma Aldrich, USA.

2.2. Bacterial strains

Table 1. *Bacterial strains used in this study.*

Strain	Plasmid*	Reference**
Staphylococcus aureus RN4220	pAT19	Vesterlund et al. 2004
Escherichia coli K12	pCGLS11	Vesterlund et al. 2004
Acinetobacter baylyi ADP1	pBAV1K-T5-LUX	Santala et al. 2016
Pseudomonas putida	pBAV1K-T5-LUX	This study

*) the plasmid carrying a bacterial luciferase operon

**) reference for the construction of biosensor strain

2.3. Cultivation

64 The bacterial strains (Table 1) were cultivated on lysogeny agar (LA) plates containing tryptone 10 g/L; yeast
65 extract 5 g/L; NaCl 10 g/L, 100 mM PB (phosphate buffer; pH 7.0; total K_2HPO_4 9.3 g/L; KH_2PO_4 6.3 g/L) and
66 agar 15 g/L. *A. baylyi* and *P. putida* strain were grown on media supplemented with 50 µg/mL kanamycin, *E.*
67 *coli* on ampicillin (100 µg/mL) and *S. aureus* on erythromycin (5 µg/mL) to maintain plasmids. *A. baylyi*, *P.*
68 *putida* and *E. coli* were cultivated in 30 °C and *S. aureus* in 37 °C.

69 New cultivation plates were prepared weekly and liquid cultivations daily. For liquid cultivations, similar
70 lysogeny broth (LB) media was used, but without agar and without PB for *P. putida*. A single colony was
71 inoculated into each of the sterile tubes containing the described media. The liquid cultivations were
72 incubated overnight in 30 °C and 300 rpm shaking.

73 2.4. Construction of *P. putida* biosensor strain

74 The plasmid pBAV1k-T5-LUX was a kind gift from Ichiro Matsumura (Addgene plasmid # 55800, Bryksin &
75 Matsumura 2010).

76 *P. putida* (DSM 291) was cultivated on LA plates (16 h, 30 °C) and in LB medium for overnight culture (30 °C,
77 300 rpm). The previously described transformation protocol (Meinhardt 2002) was used with modifications.
78 In brief, LB was used for cultivation. The cells (optical disturbance at 600 nm [OD] 0.7) were harvested by
79 centrifugation (11200 x g, 2 min) and washed three times with ice cold glycerol (10 %). The *P. putida* cells
80 were electroporated with BIO-RAD Micropulser™ (Bio-Rad Laboratories Inc., USA). Prewarmed LB was added
81 after the electroporation, the cells were revived for 90 min in 30 °C and plated for cultivation on selective LA
82 plates.

83 2.5. Sundew propagation and extraction

84 Field-grown sundews were collected from two peatlands (Peatland 1; Lehtolamminneva N 62°6.01' E
85 22°57.22', and Peatland 2; Kivineva N 61°57.77' E 23°23.98') in Western Finland. The vegetatively reproduced
86 sundew tissue was initiated from a small sundew seedling which was multiplied on half-strength Murashige-
87 Skoog nutrient agar medium (Murashige & Skoog 1962). It was stored in freezer until extraction. Sundew
88 plants and tissues were extracted with 100 % ethanol (EtOH; 0.15 g fresh weight plant tissue/mL EtOH) after
89 grounding in a mortar.

90 The extracts were air- and N_2 dried. The extracts were left to evaporate in open vials in a fume hood in room
91 temperature for 6 days. To speed the drying process, N_2 gas was led into the vials using a separate outlet for
92 each vial for 4—6 hours, until all EtOH was removed. The dried extracts were stored in -20 °C and dissolved
93 into sterile double distilled water (DDW). The sample dilutions were stored in -20 °C between the test runs
94 and new dilutions prepared weekly.

2.6. Test assay runs

For the validation of the developed biosensor cell panel, the effects of EtOH, naphthoquinones and quercetin were tested. Concentrations of 0.33, 1.7, and 17 v/v-% (volume percentage) per well were used, prepared by diluting EtOH in DDW. DDW was used as a blank control sample. For juglone, the tested concentrations per well were 3.1, 6.3, 13, 25 µg/mL; for lawsone 43, 85, 170, 340 µg/mL; for plumbagin 0.13, 0.26, 0.52, 1.0 µg/mL; and for quercetin 5.0, 10, 20, 100 µg/mL. Fresh dilutions of naphthoquinones and quercetin were prepared weekly. The sundew extracts were tested in concentrations with 0.2, 0.4, and 0.8 mg/mL of plant material dry weight per well.

An aliquot of 50 µL of the samples were pipetted in triplicate into the wells of a 96-well, opaque white plate (Corning, USA). Liquid cultivation (OD 1.3—2.0 depending on the species), diluted 1:1 in fresh LB (100 µL) was added to the wells. The bioluminescence measurement was started immediately. A new measurement was done every 5 min until 90 minutes of incubation time had passed with Fluoroskan Ascent FL (Thermo Scientific, USA) microplate reader (room temperature).

The results are represented in Figures 1—3 as inhibition-% (inhibition percentages). They were calculated as the percentage change of the sample wells' average from the average of blank wells after an incubation time, here 50 min. Negative change indicates an increase of signal in sample wells compared to the blank wells, whereas positive inhibition-% indicates a decrease in signal. The error bars in Figures 1—3 represent the CV-% (coefficient of variation) of the sample wells. The SNR (signal-to-noise-ratios) were calculated by dividing the highest signal of microplate at 50-minute measurement by previously determined average signal of two medium-only samples (data not shown).

3. Results and discussion

3.1. Initial validation of panel

First, it was confirmed that all strains produce a measurable light signal. The SNRs in increasing order were *S. aureus* (8.78), *P. putida* (99.9), *A. baylyi* (1685) and *E. coli* (5 996), indicating the order of their ranges as well. Although the pAT19 plasmid carrying bacterial luciferase operon is optimized for *S. aureus* (Vesterlund et al. 2004), the other strains produced clearly higher SNRs in the studied conditions.

Then, the functionality of the biosensor strains was tested with EtOH. Based on the time resolved data (Supplementary Figures 1-4), the time point of 50 min incubation was chosen to ensure adequate reaction time for the different species. As expected, the inhibition response was dose-depended, as increasing ethanol concentrations caused increasing inhibition (Figure 1, showing results after 50 min of incubation).

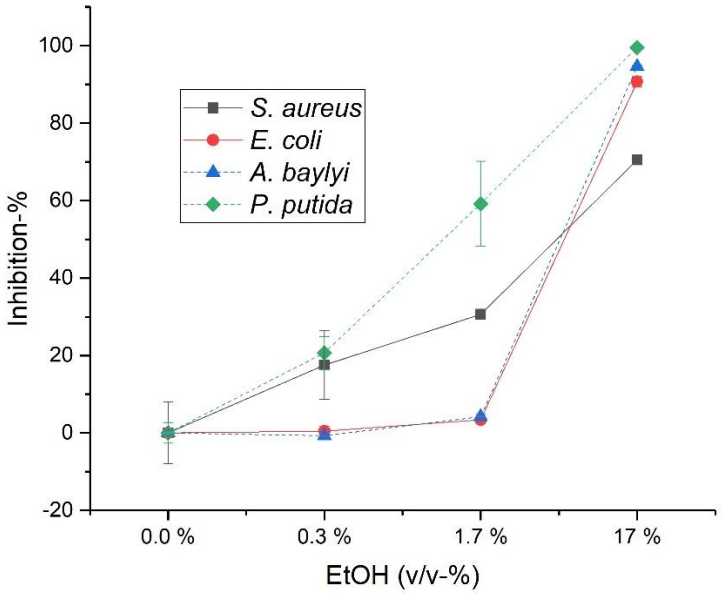

Figure 1. *Inhibition percentages (-%) for biosensor panel strains incubated with EtOH after 50 min of incubation. Error bars represent CV-%, v/v-% is percentage by volume.*

The inhibition-% *of E. coli* and *A. baylyi* biosensors caused by 0.3 % and 1.7 % EtOH were 0.5, 3.4, -0.7 and 4.2 % respectively. For example, *A. baylyi* can utilize EtOH as a carbon source and 0.38 % concentration has been used for cultivations (Salcedo-Vite et al. 2019). However, *S. aureus* and *P. putida* were more sensitive towards ethanol*:* both sensors showed approximately 30 inhibition-% after 50 min of incubation with 0.3 %.

3.2. Testing the panel with pure plant metabolites

The panel was used to measure inhibition caused by plant metabolites, which have previously demonstrated antibacterial effects. The metabolites included both naphthoquinones and quercetin (Paper et al. 2005; Jaisinghani 2017), a flavonoid. The tested naphthoquinones were juglone, lawsone and plumbagin (Mahapatra et al. 2007; Wang et al. 2016; Rahmoun et al. 2012; Paiva et al. 2003). Previously reported minimal inhibitory concentrations (MICs) of the metabolites are found in Supplementary Table 1.

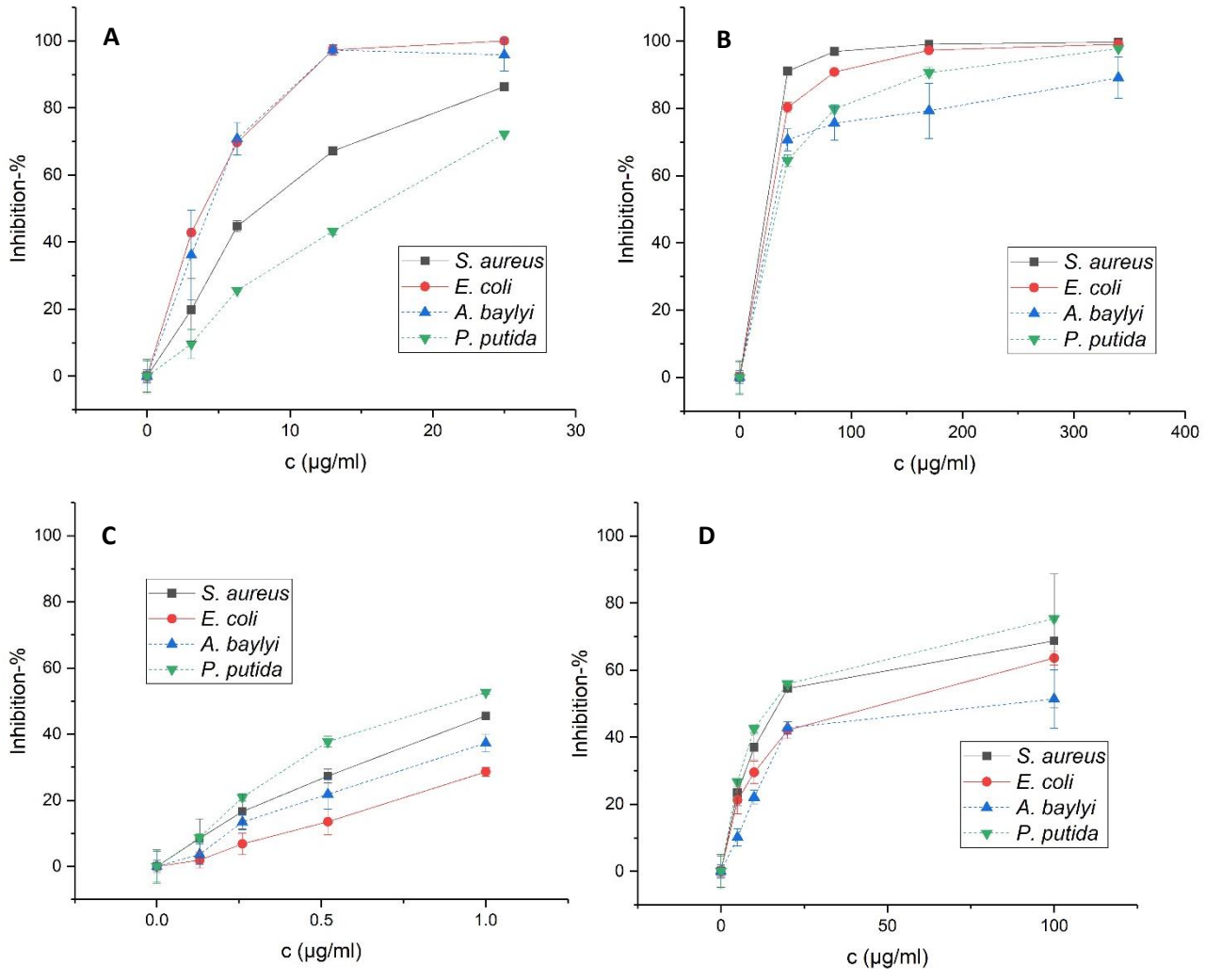

Figure 2. *Inhibition percentages (-%) caused by naphthoquinones and quercetin after 50 min of incubation. A) Juglone. B) Lawsone. C) Plumbagin. D) Quercetin. Error bars represent CV-%.*

Our biosensor panel gave a comparable result with the reported MIC value for juglone (Figure 2A), where 25 µg/mL inhibited *S. aureus* by 86 %. However, differences are seen in lawsone (Figure 2B) tests, for example *E. coli* sensor was inhibited 91 % by only 170 µg/ml of lawsone, suggesting that less than 512 µg/ml is needed to fully inhibit the cellular processes of *E. coli*. On the other hand, 1.0 µg/ml of plumbagin (Figure 2C) and 20 µg/ml of quercetin (Figure 2D), comparable concentrations to the previously reported MICs, only inhibited *S. aureus* in our panel by approximately 50 %. However, it should be noted that MIC tests measure the lowest concentration that inhibits bacterial growth (Wiegand et al. 2008), while the developed panel measures the toxicity effects towards the bioluminescence production.

Each of the bacterial strains in the panel showed a unique response to the pure plant metabolites. This could be caused by various factors, such as the cell wall differences, which affect for example the transfer of substances into the cells. A G+ (Gram positive) species, *S. aureus,* has two cell wall layers, as opposed to one

layer of G- (Gram negative) bacteria (Vollmer & Seligman 2010). Thus, the biosensor panel helps in the assessment and differentiation of the substances' effects on the bacteria.

3.3. Demonstrating the panel's functionality with sundew extracts

As a proof of concept, antibacterial effects of sundew (*Drosera rotundifolia*) extract, which is known to contain antibacterial flavonoids such as quercetin (Paper et al. 2005), were tested by the biosensor panel. The biosensor strains reacted differently to the sundew extracts (Figure 3).

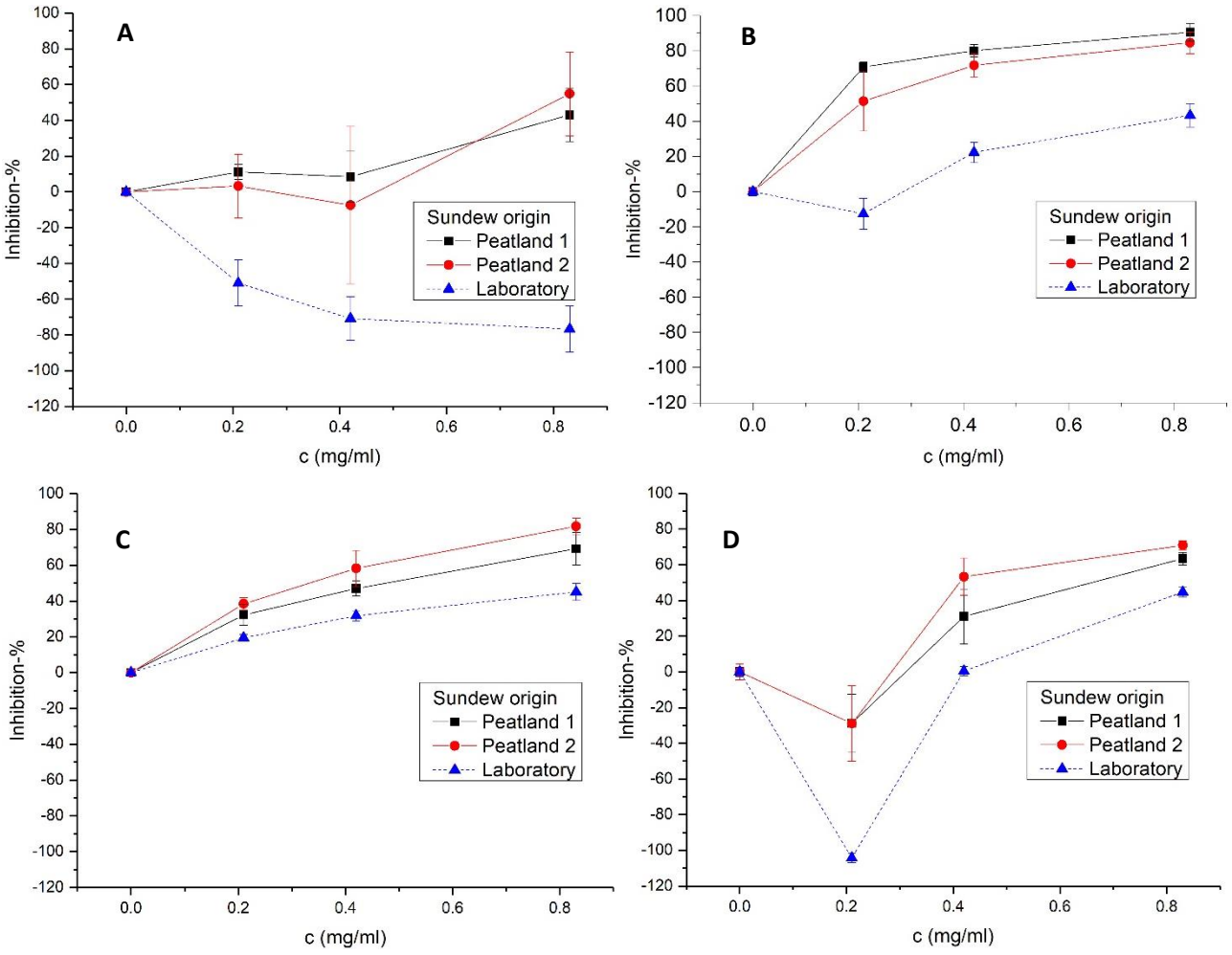

Figure 3. *Inhibition percentages (-%) caused by Drosera rotundifolia extracts for biosensor panel strains after 50 minutes of incubation. Error bars represent CV-%. A) S. aureus. B) E. coli. C) A. baylyi. D) P. putida.*

No significant differences ($P > 0.1$, Student's unpaired t test for mean of all concentrations) could be detected with *P. putida* biosensor between the sundew extracts from the two peatlands (Figure 3D). The extract from vegetatively propagated sundew tissue showed lower inhibition-% than Peatland 1 ($P < 0.05$ for all concentrations) and Peatland 2 ($P < 0.01$ for all concentrations). The effects of peatland sundew extracts were dose dependent in the whole panel, except of the effect on *S. aureus* (Figure 3A) at 0.42 mg/mL. The most sensitive species to field-grown sundew extracts was *E. coli* (Figure 3B) as even 0.21 mg/mL inhibited

the growth approximately by 80 %. *P. putida* was not inhibited but stimulated by 0.21 mg/mL and was also overall the most tolerant of the G- bacteria.

The extracts of laboratory grown sundew caused a different response compared to the field-originating extracts. *A. baylyi* (Figure 3C) showed dose dependent inhibition to the laboratory sundew extracts, but 0.83 mg/mL caused approximately as much inhibition as 0.21 mg/mL of the peatland sundew extract (40 %). *S. aureus* was stimulated to produce light by all tested concentrations. *P. putida* and *E. coli* were stimulated by 0.21 mg/mL. The luminescent light signal of those *P. putida* wells was over 2-fold compared to that of the blank sample (0.0 mg/mL). With extract sample dilution, the inhibitory compounds are also diluted, which gives the bacteria a chance to benefit from the sugars and other nutrients potentially present in the extracts. Sequentially, this can cause higher light signal than in the control wells with no extract.

4. Conclusion

Our study demonstrates that a panel of bioluminescent bacterial whole-cell biosensors can be used to find species specific antibacterial responses to plant extracts, which are not revealed when using only one species for screening. Differences between *S. aureus* and the G- strains were detected both in the performance and the response to sundew extracts. Although both *A. baylyi* and *P. putida* are soil bacteria, *P. putida* was more tolerant to the sundew extracts and juglone. This highlights the benefits of a panel of bacterial biosensor strains for the screening of antimicrobial substances. The panel proposed here suggests a combination of bacteria, which are suitable for high-throughput screening of plant extracts using 96-well plate format, with a rapid test protocol of under 2 hours. If a need arises to study other pathogens, they could easily be applied to the panel by introducing the bioluminescence operon. The panel presents an efficient, new method for future studies to screen potential antibacterial substances and plant extracts.

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