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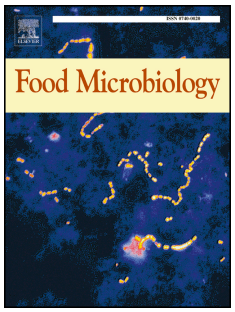

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Vacuolar localisation of anthocyanin pigmentation in microgreen cotyledons of basil, cabbage and mustard greens does not impact on colonisation by Shiga-toxigenic *Escherichia coli* O157:H7.

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Running title

No impact of anthocyanin on *E. coli* O157:H7 colonisation of microgreens

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Abstract

Microgreens, the immature plants harvested after a few weeks of growth, are perceived as a heathy, nutritious food ingredient but may be susceptible to colonisation by human pathogens including Shiga-toxigenic *Escherichia coli* (STEC). Some microgreen cultivars accumulate anthocyanins or secrete essential oils which, when extracted or purified, have been reported to inhibit bacterial growth. Therefore, the impact of anthocyanins on bacterial colonisation by STEC (Sakai) was compared for three species that have pigmented cultivars: basil (*Ocimum basilicum* L.), cabbage (*Brassica oleracea* L.) and mustard greens (*Brassica juncea* L.). Inoculation with low concentrations of STEC (Sakai) (3 $\log_{10}$ colony forming units/ml (CFU/ml)) during seed germination resulted in extensive colonisation at the point of harvest, accumulating to ~ 8 $\log_{10}$ CFU/g FW in all cultivars. Bacterial colonies frequently aligned with anticlinal walls on the surface of epidermal cells of the cotyledons and, in basil, associated with peltate and capitate gland cells. Crude lysates of pigmented and non-pigmented basil

cultivars had no impact on STEC (Sakai) growth rates, viability status or biofilm formation. Anthocyanins are located within plant vacuoles of these microgreen cultivars and did not affect colonisation by STEC (Sakai) and pigmentation therefore cannot be considered as a controlling factor in bacterial interactions.

KEYWORDS

Microherb, fresh produce, foodborne pathogen, vegetable

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1 INTRODUCTION

'Microgreens', 'microleaf' and 'microherbs' are marketing terms for immature plants, grown in light and harvested less than a month after germination (Di Gioia et al., 2015). Whilst often consumed in Asian cultures, they are increasingly popular as ingredients in Western countries, providing colour and intense flavour, and may have enhanced nutritional and potentially nutraceutical value (Kyriacou et al., 2016). The method of growth of microgreens can be favourable for proliferation of food-borne pathogens including Shiga-toxigenic *Escherichia coli* (STEC) (Riggio et al., 2019; Wright and Holden, 2018) similar to the situation for sprouted seeds. Several STEC outbreaks have been associated with sprouted seeds (Buchholz et al., 2011; CDC, 2016) and STEC can interact with plants as alternative hosts (Holden et al., 2009; Méric et al., 2013). STEC has the potential to colonise a range of microgreen species to high levels under commercially relevant growth conditions (Wright and Holden, 2018). In addition to the public health drivers, foodborne illness incurs large financial costs, whether at the retail or national levels (Bartsch et al., 2018; Hussain and Dawson, 2013).

The red colouration of some cultivars of plant species including basil (*Ocimum basilicum* L.), cabbage (*Brassica oleracea* L.) and mustard greens (*Brassica juncea* L.) is due to the presence of anthocyanins. These and other flavonoids have a role in protecting plants from abiotic stresses like high light intensities and UV-B radiation and in protection against phytopathogens (Harborne and Williams, 2000; Tattini et al., 2017). The synthesis of anthocyanins is understood to be localised on the cytosolic side of the endoplasmic reticulum, but they are primarily located in the vacuole, where the pH influences the resulting colour. Anthocyanins have been the subject of much recent interest into their pharmacological potential as antioxidant, anti-inflammatory and antimicrobial agents (Liu et al., 2021). In particular, they are under investigation by the food industry as natural colourants, flavouring, antioxidant or antibacterial agents to replace synthetic chemical additives or preservatives (Demirdöven et al., 2015). Here, we tested whether pigmentation from anthocyanins impacted the colonisation of microgreens during propagation, by the foodborne pathogen STEC O157:H7. Different green, or red, anthocyanin-producing, cultivars of three species, basil ('Genovese' and 'Dark Opal'), cabbage ('Golden acre' and 'Red Drumhead') and mustard greens ('Wasabini' and 'Red Carpet'), were inoculated with an STEC isolate (Sakai), previously associated with large-scale disease outbreaks from contaminated white radish sprouts (Michino et al., 1999). The experimental set-up was designed to mimic commercial growth hydroponic conditions, and the plants harvested at a suitable size for microgreen consumption.

2 MATERIAL and METHODS

2.1 Bacterial strain and growth conditions

Escherichia coli O157:H7 Stx-negative strain Sakai was used for biosafety reasons, which contains an insertional inactivation of *stx2* with a kanamycin cassette and partial deletion of the *stx1a* coding sequence and 5' region (Dahan et al., 2004). It was transformed with the plasmid-borne GFP fluorescent reporter *pgyrA-gfp* (termed STEC (Sakai)) was used for visualisation and for quantification (for details see Wright and Holden, 2018). Bacteria were routinely cultured for ~18 h in lysogeny broth (LB) at 37°C with aeration, and prior to use sub-cultured 1:100 in rich defined MOPS (RDM) glucose and grown at 18°C for ~20 h, in the presence of chloramphenicol (25 µg/ml). Long-term stocks were stored at -80°C in 20 % glycerol.

2.2 Plant material

Seed of the following red or green plant cultivars (cvs.) were used: basil (*Ocimum basilicum*) cvs. Micro Leaf Basil 'Dark Opal' (M) and 'Genovese' (C); cabbage (*Brassica oleracea*) cvs. 'Red Drumhead' (C) and 'Golden Acre' (D), mustard greens (*Brassica juncea*) cvs. 'Red Carpet' (C) and 'Wasabini' (S); purchased from Marshalls seeds, Huntingdon (M), Chiltern Seeds, Wallingford (C), Sarah Raven Seeds, Wiltshire (S), or Dobies, Paignton, UK (D).

2.3 Seed sterilisation, plant growth and inoculation with bacteria

Seeds were treated with 5 % Domestos (Unilever: includes 10 % sodium hypochlorite, 0.1-1 % sodium hydroxide and surfactant) diluted in sterile distilled water (SDW) for 5 min followed by 6 rinses in SDW. Under sterile conditions, seeds were transferred to round (7.5 cm diameter x 8 cm high) polypropylene containers lined with dry matting (GrowFelt Purple, CN Seeds Ltd., Ely, UK). Seed surface sterilisation reduces endemic microbes and helps to standardise conditions. STEC (Sakai) were diluted to OD_{600} of 0.2 (~8.0 $\log_{10}$ CFU/ml) in 0.5 x Murashige and Skoog medium (Murashige and Skoog, 1962) including vitamins adjusted to pH 5.8 with NaOH (Duchefa, M0222); the same medium required to maintain plant growth and reduce the variable factors to 'bacterial inoculant' only. They were further diluted to 3.0 $\log_{10}$ CFU/ml in 0.5 x MS prior to seed inoculation. The matting was moistened with 15 ml of bacterial suspension, and the containers fitted with lids incorporating a sun cap closure (Sigma-Aldrich S5939) to allow gas exchange. They were then transferred to a growth cabinet (16 h light, 22.8 ± 0.02°C, humidity 96.5 ± 0.05%; 8 h dark, 20.6 ± 0.01°C, humidity 99.2 ± 0.04%) until the time of harvest. All containers, matting or plant growth media were sterilised by autoclaving prior to use. For quantification, plants were cut just above the crown to harvest the edible portion of tissue, weighed and macerated with a pestle and mortar in 1 ml phosphate buffered saline (PBS). These extracts were serially diluted 10-fold in PBS, with 4 replicate 10 µl aliquots

plated as micro-drops on to 24.5 cm square bioassay plates containing MacConkey agar with chloramphenicol (Pious et al 2015), and incubated overnight at 37°C. Resultant colonies were expressed as CFU/g of fresh weight (FW) with a detection limit of 403 CFU/g FW (=2.6 $\log_{10}$ CFU/g FW or 7 CFU/g $FW^{1/3}$), based on presence of at least 1 bacterial colony in one of four samples and converted by the maximum plant weight. For each species, five containers were sown with either the red or green cultivar, with one plant being sampled from each container at each harvest date, and the experiment was repeated twice, independently. Due to the different growth rates, basil was harvested between 6 and 9 days, and cabbage and mustard between 5 and 8 days from sowing.

2.4 Leaf lysate extraction and STEC (Sakai) inoculation

Basil cultivars 'Dark Opal' and 'Genovese' were germinated in standard compost and grown at ~ 21 °C in a glasshouse. Two weeks after germination, the leaves were aseptically harvested using sterilised scissors and macerated to a fine powder in liquid nitrogen. 10 g leaf powder was added to 40 ml SDW, vortexed for 1 min and harvested by centrifugation (4,000 g for 20 min). The supernatant was filter sterilised and stored at -20 °C. Leaf extracts were used to supplement RDM medium with or without 0.2 % (v/v) glycerol, at a concentration of 40 % (v/v), as previously (Crozier et al., 2016). To determine growth rates, 2 ml of STEC (Sakai) were harvested by centrifugation (4,000 g for 5 min) and re-suspended in 2 ml RDM, or in LB as a control. The cell density was measured and adjusted to a final OD_{600} of 0.05 and 200 µl was added per well to a multiwell plate (Honeycomb; Thermo Fisher, USA), in sample reps of 4. The cultures were incubated at 21 °C for 60 hours and cell density read in a prewarmed plate reader (Bioscreen C; Oy Growth Curves Ab Ltd., Finland), with measurements taken every 15 min, and the multiwell plates shaken for 60 s pre- and post-measurement. The results were exported from the plate reader proprietary software as tab-delimited files. To determine the cell viability status, bacteria were grown as above, incubated at 21 °C for 60 hours, serially diluted 10-fold in PBS and plated onto MacConkey plates, half of which contained 0.1 % Na-pyruvate to resuscitate stressed, non-culturable cells (Mizunoe et al., 1999), and incubated at 37 °C for 24 hours. The colony number were converted to $\log_{10}$ CFU/ml. Biofilm formation was assessed from cells grown as above, with 200 µl per well added at a density of OD_{600} 0.02 and incubated at 21 °C for 60 hours. Biofilms were assessed by crystal violet staining, as described previously (Merget et al., 2019).

2.5 Confocal microscopy

Cotyledons were harvested and mounted in SDW on microscope slides under a coverslip held in place using double-sided tape and observed using a Nikon A1R confocal laser scanning microscope mounted on an NiE upright microscope fitted with an NIR Apo 40x 0.8W water

dipping lens and GaAsP detectors. GFP (green) and chlorophyll (blue) were excited at 488 nm with emissions gathered at 500-530 nm and 663-738 nm respectively Anthocyanin was excited sequentially at 561 nm with emissions gathered at 570-620 nm (magenta). Images are false colour single confocal fluorescence sections or maximum intensity projections, produced using NIS-elements AR software. Colour plates were prepared using Photoshop CS5 software using enhancement of levels where appropriate.

2.6 Statistical analysis

Differences between green and red cultivars for the three species were analysed using linear mixed effects (LME) models. In order to normalise the response variable, CFU/g FW plant tissue, a cube root transformation was used (Shapiro-Wilk Normality test, $W = 0.997$, $p = 0.951$) as the usual $\log_{10}$ transformation failed to meet the normality assumption ($W = 0.831$, $p < 0.0001$). The fixed effects were cultivar as a factor and age, defined as day from sowing, as a continuous variable. The addition of a fixed effect for experiment had no significant effect for any species ($p < 0.6$). As replicate experiments were undertaken for each species, and plants were taken from the same five pots at each sample age, pot nested within experiment was used as a random effect. Based on lower Akaike Information Criterion (AIC) score, inclusion of this random effect structure was justified compared to a general linear model. The LME models were implemented using LMER in the package LME4, R4.21.

Bacterial growth rates were determined by fitting to the Gompertz model using DMFit (ComBase) add-in for Excel to obtain the maximum growth rate (μmax) and the maximum cell density (y_max). ANOVA was run to determine differences in viability status and biofilm formation.

3 RESULTS

3.1 Localisation and colonisation dynamics of STEC (Sakai) on microgreens

To first determine whether red-pigmented cultivars exhibited altered localisation of STEC, seeds of different green or red cultivars of three species; basil, cabbage and mustard greens, were inoculated with relatively low concentration of STEC (Sakai) (3 $\log_{10}$ CFU/ml) during germination and examined at the 'microgreen' stage. There were no observable differences between the cultivars in that the seedlings became extensively colonised, with the majority of bacteria being located on the epidermal cell surfaces (Fig. 1). Bacteria were observed in close proximity to the gland cells of basil ('Genovese' Fig. 1a and b) and ('Dark Opal' Fig. 1c and d) and aligned with the anticlinal walls of cabbage ('Golden Acre' Fig. 1e, 'Red Drumhead' Fig. 1f) or mustard greens ('Wasabini' Fig. 1g or 'Red Carpet' Fig. 1h).

The number of STEC (Sakai) colonising each cultivar was quantified over four days, starting five days (cabbage and mustard greens) or six days (basil) from sowing. Germination of seeds on matting watered with relatively low concentrations of bacteria resulted in extensive colonisation of the cotyledons to around 8.0 $\log_{10}$ CFU/g FW (464 CFU/g $FW^{1/3}$ Fig. 2). There was no significant difference in CFU/g FW between basil and cabbage (p=0.575), in a model where cultivar was nested in species, but mustard differed with respect to intercept and slope with days from sowing (Table 1). In general, the level of colonisation decreased with time from the start of sampling at day 5 post-sowing (day 6 for basil) until the end of the experiment (Fig. 3), although this trend was only significant in cabbage when considered in a single species model (p=0.041). There was no significant difference between the green and red cultivars for any species either when considering the full model or for species models in which the effect of days from sowing and cultivar were compared (p > 0.27).

3.2 Leaf lysate extracts from purple basil do not inhibit STEC growth, viability or biofilm formation

As there was no apparent antimicrobial impact for intact, growing plants, the bacterial growth response was tested in crude extracts, using basil as a representative species. The impact of both basil cultivars was tested on STEC growth and viability, using freeze-dried leaf lysate extracts that were used to supplement defined media. Extracts from either basil cultivar supported growth of STEC (Sakai) at 21 °C (Fig. 4a), with no obvious difference between the rates for either cultivar in the base RDM medium. Although growth rates were faster in RDM glycerol medium supplemented with 'Dark Opal' extract compared to 'Genovese' extract, the difference was marginal (0.020 'vs' 0.018, respectively). Growth rates and maximum cell densities were similar to that for LB medium, but cultures in media that lacked glycerol reached only 35 – 38 % of the cell density of cultures grown in media containing glycerol. The viability

166 status of the cells (as determined from resuscitation medium) was not significantly affected
167 during growth in the extracts compared to viable plate counts, as measured at 24 hours
168 (p=0.6177) or 60 hours (p=0.445) at 21 °C, or after incubation of 25 days at 21 °C in either of
169 the neat (100 %) extracts (p=0.496). Neither of the basil cultivar extracts particularly enhanced
170 or suppressed biofilm formation, as measured after 60 hours at 21 °C, in media with or without
171 glycerol (Fig. 4b: p=0.057).

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4 DISCUSSION

Although concentrated, extracted anthocyanins possess antibacterial properties, we show that such activity did not occur *in planta* since pigmented cultivars of three microgreen species were colonised equally well by the foodborne pathogen, STEC O157:H7 (isolate Sakai) as the green, non-pigmented cultivars of the same species. Extracted anthocyanins have been reported to have beneficial health effects including antimicrobial activities (Khoo et al., 2017). For example, proanthocyanidins isolated from cranberry show bacterial anti-adhesion activity against strains of uropathogenic P-fimbriated *E. coli* (Howell, 2007) whilst anthocyanins, incorporated into dental copolymer, are being tested as natural antibacterial agents against *Streptococcus mutans* (Hrynash et al., 2014). *In planta* anthocyanins are compartmented primarily in the vacuoles of the epidermal cells (Zhao and Dixon, 2010) so that bacteria are unlikely to encounter them unless the plant cells are damaged. This lack of exposure of STEC bacteria to anthocyanins, together with the concentrated levels in extracts, may therefore explain the lack of any impact on *in planta* colonisation for the pigmented compared to the non-pigmented varieties. Furthermore, the effect was consistent across different pigmented plant species. Since red or green cultivars tested are genetically distinct, albeit in the same species, there are additional factors that could impact colonisation.

Multiple other plant-derived secondary metabolites may also show antimicrobial activity, including essential oils from basil (Sakkas and Papadopoulou, 2017). Therefore, crude aqueous soluble extracts of basil were assessed, which from the 'Dark Opal' cultivar potentially include oils secreted by the gland cells as well as anthocyanins released from the vacuoles. However, neither extract of either purple pigmented or green non-pigmented basil impacted STEC (Sakai) growth, viability status or biofilm formation. Similarly, a clinical isolate of *Salmonella enterica* serovar Senftenberg was equally unaffected by growth on (green) basil, exhibiting resistance to basil oil and its components (Kisluk et al., 2013). The extracts used here were not concentrated or purified in contrast to forms investigated by others for antimicrobial activity (Burt, 2004; Suppakul et al., 2003), which may in part at least, explain the effect. The structure of the gland cells on the cotyledons of both green and red basil appeared essentially similar to those observed on young and more mature basil leaves, and the aroma released during extract preparation was indicative of some oil production. STEC (Sakai) were observed growing in close proximity to both the 2-celled capitate and 4-celled peltate gland cells involved in the synthesis and storage of phenylpropenes that are components of the essential oils secreted by basil (Gang et al., 2001; Werker et al., 1993). This suggests that oils potentially secreted do not influence bacterial colonisation and distribution. Instead, the recessed positioning of the gland cells may offer a protected microclimate for growth of the STEC bacteria, similar to the stomatal pores and channels

formed above anticlinal walls of the epidermal cells (Monier and Lindow, 2004), as shown here by colonisation of cabbage or mustard greens by STEC. This distribution on the surface of, and occasionally within, cotyledons is similar to previous observations of basil and other species including amaranth and broccoli (Wright and Holden, 2018). The decrease in numbers of colonising bacteria with age, also observed previously in broccoli (Wright and Holden, 2018), may be the result of decreased nutrient availability on the cotyledons. However, for microgreens where the cotyledons are consumed, this is unlikely to reduce the potential risk at the point of consumption.

4.1 Conclusion

The cotyledons of numerous species consumed as microgreens, including basil, cabbage and mustard greens, have the potential for extensive colonisation by STEC (Sakai) and, *in planta*, intracellular anthocyanins do not play any antimicrobial role reducing their incidence. This raises the need for microbial risk management for consumption of microgreens as raw products, pigmented or not, in line with that for sprouted seeds. Risk management also needs to take account of innovative technologies used for plant propagation, which lend themselves to this type of product. The information can be incorporated into existing microbial risk guidance for growing fresh produce.

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6 CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

7 FIGURES AND TABLES

FIGURE 1 Colonisation of microgreens by STEC (Sakai)

STEC (Sakai) expressing GFP close to peltate (P), capitate (C) gland cells or stomata (S) on the surface of cotyledons of 'Genovese' (**a** and **b**) and 'Dark Opal' (**c** and **d**) basil seen in maximum intensity projections (**a** and **c**) or single sections (**b** and **d**). STEC overlying the anticlinal epidermal cell walls of 'Golden Acre' (**e**) 'Red Drumhead' (**f**) cabbage, 'Wasabini' (**g**) or 'Red Carpet' (**h**) mustard greens. Scale bars represent 50 µm (**a**, **c**), 10 µm (**b**, **d-h**).

FIGURE 2. Observed colonisation rates

Differences in colonisation for STEC (Sakai) plotted in relation to days from sowing for anthocyanin-pigmented (magenta) and green-pigmented (green) cultivars of three microgreen species;- basil, cabbage and mustard greens. Results from two experiments are indicated by symbols (circle and triangle).

FIGURE 3. Predicted colonisation rates.

Fitted relationship for colonisation of STEC (Sakai) in relation to days from sowing based on the full interaction linear mixed model together with lower and upper pointwise standard errors for each cultivar. The colonisation data was cube root transformed to conform to normality and homogeneity of variance assumptions.

FIGURE 4 Growth and biofilm formation of STEC (Sakai) in basil leaf lysate extracts.

(a) Maximum growth rates (Log_{10} CFU/h - X) and maximum cell densities (Y-max (OD_{600nm} - n) of STEC (Sakai) obtained from data fitted to growth models (in DMFit). Growth was quantified in a multi-well reader over 60 hours at 15-minute intervals at 21 °C. The fitted rates with standard errors of the rates are provided from three experimental replicates (n=12). Growth rates in LB were included for reference: a maximum growth rate of 0.021 (LB) equates to a generation time of 5.52 hours. **(b)** Biofilm formation was quantified from crystal violet retained measured at OD_{590nm} after 60 hours at 21 °C. The averages and variance are provided from two experimental reps (n=8). RDM and RDMg refer to rich defined MOPS medium without and with glycerol respectively; DO and G refer to leaf lysate extracts (40 %) of 'Dark Opal' and 'Genovese' cultivars respectively.

TABLE 1. Linear Mixed Model of colonisation ($CFU.gFW^{1/3}$) in relation to two factors; species and cultivar (red or green) and the continuous variable, days from sowing. Estimates, confidence interval and significance values given for the fixed effects are presented, together with R^2 of model fit.

Predictors	*Estimates*	*Confidence Intervals*	*p*
(Intercept)	594.70	387.50 – 801.89	**<0.001**
Cabbage	80.92	-201.78 – 363.62	0.575
Mustard	327.94	45.24 – 610.64	**0.023**
Days from sowing	-42.87	-63.47 – -22.28	**<0.001**
Basil * Red	34.95	-11.1 – 81.00	0.137
Cabbage * Red	38.92	-7.13 – 84.98	0.098
Mustard * Red	-44.52	-90.57 – 1.53	0.058
Cabbage * Days from sowing	4.94	-24.19 – 34.06	0.740
Mustard *Days from sowing	-34.90	-64.03 – -5.78	**0.019**
Observations	240		
Marginal R^2 / Conditional R^2	0.345 / 0.558		

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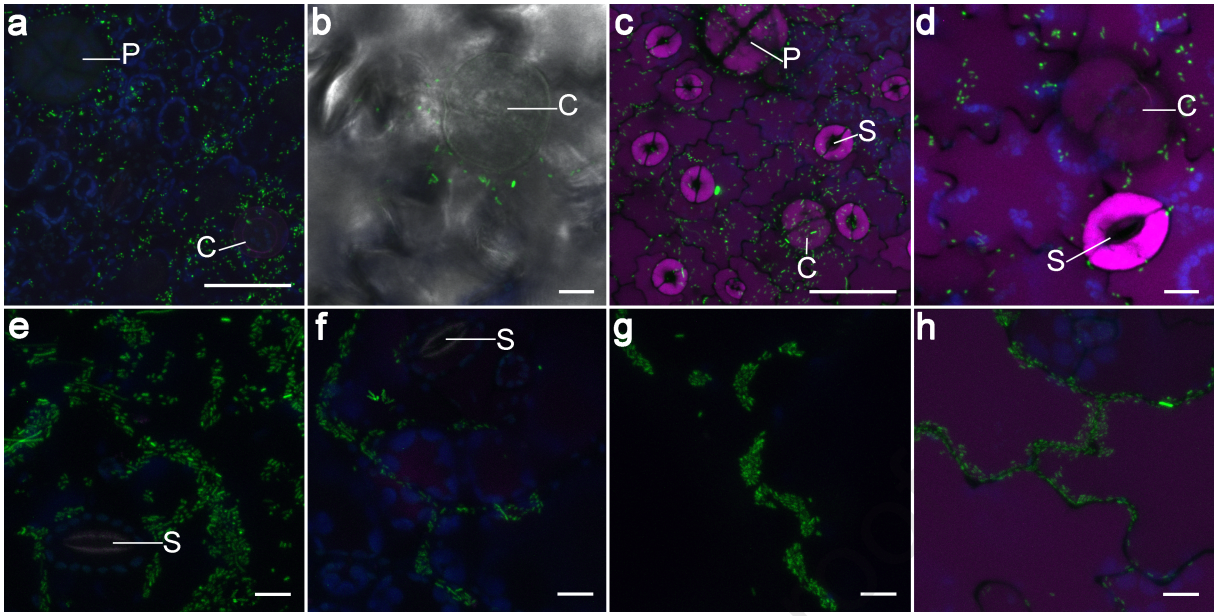

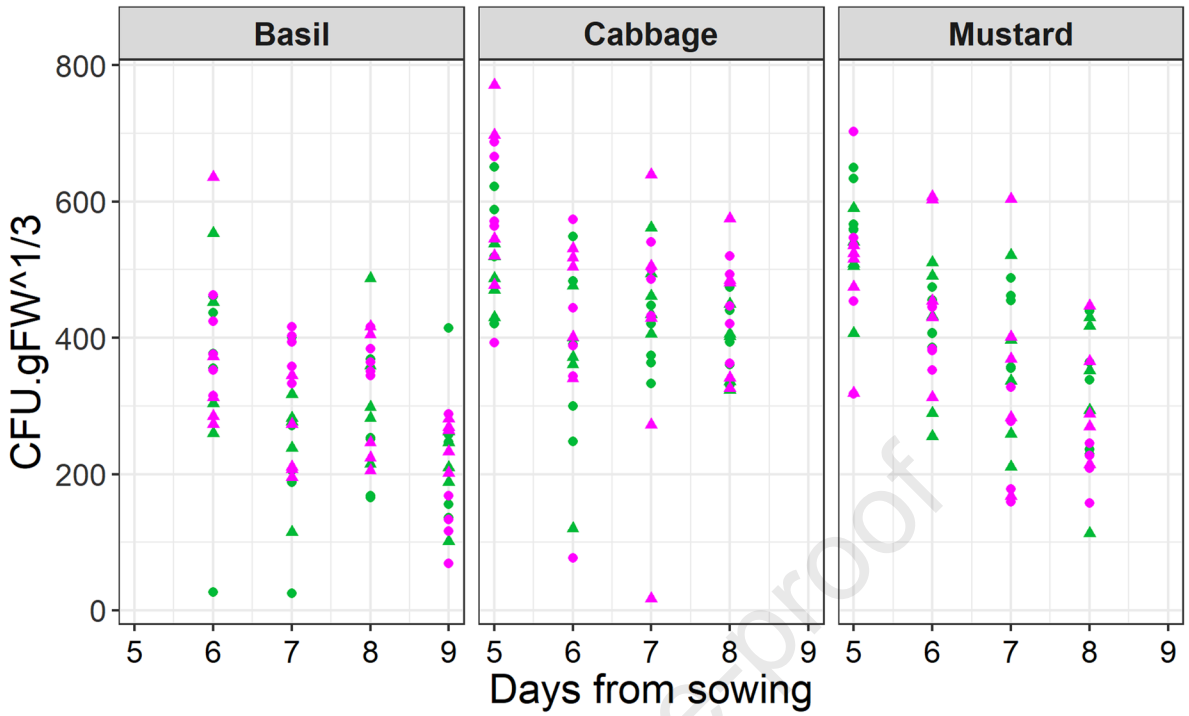

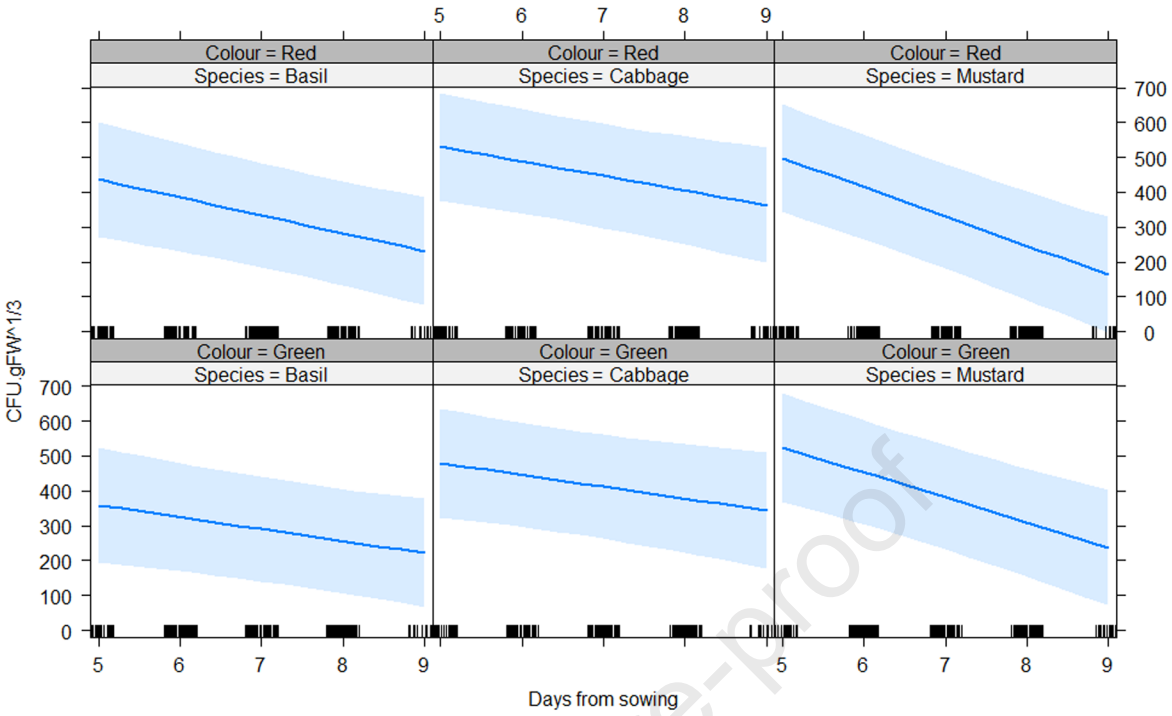

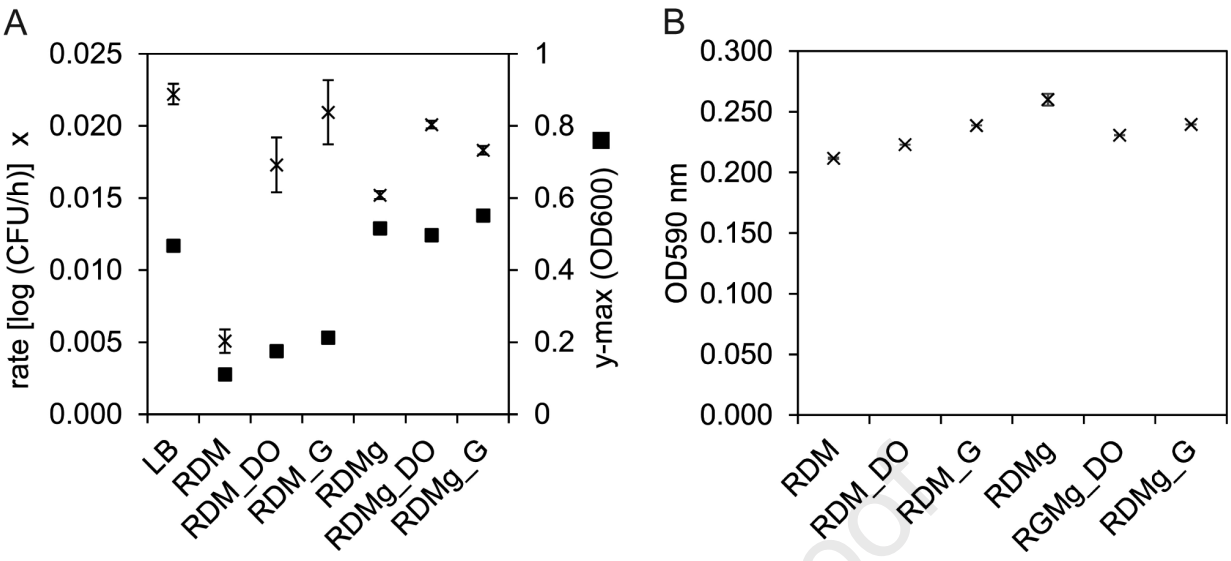

Vacuolar localisation of anthocyanin pigmentation in microgreen cotyledons of basil, cabbage and mustard greens does not impact on colonisation by Shiga-toxigenic *Escherichia coli* O157:H7.

Kathryn M. Wright [a], Jacqueline Marshall [a], Peter J. Wright [b], Nicola J. Holden [a c]*

Each Highlight can be no more than 85 characters, including spaces

- Microgreens are susceptible to contamination by foodborne bacterial pathogens
- Many microgreens or microherbs are naturally coloured, or pigmented
- *E. coli* O157 grew equally well on microgreens with or without pigments
- *E. coli* O157 was also unaffected by crude extracts of basil microgreens
- Pigmentation in microgreens does not imply a reduced risk of foodborne pathogens

Anthocyanin pigmentation in microgreen cotyledons does not impact on colonisation by Shigatoxigenic *Escherichia coli* O157:H7.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.