Methylobacterium, a major component of the culturable bacterial endophyte community of wild *Brassica* seed

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Abstract

Background. Plants are commonly colonized by a wide diversity of microbial species and the relationships created can range from mutualistic through to parasitic. Microorganisms that typically form symptomless associations with internal plant tissues are termed endophytes. Endophytes associate with most plant species found in natural and managed ecosystems. They are extremely important plant partners that provide improved stress tolerance to the host compared with plants that lack this symbiosis. Fossil records of endophytes date back more than 400 million years, implicating these microorganisms in host plant adaptation to habitat transitions but it is only recently that they have received attention from the scientific community. Plant domestication has reduced endophyte diversity and therefore the wild relatives of many crop species remain untapped reservoirs of beneficial microbes. *Brassica* species display enormous diversity and subsequently provide the widest assortment of products used by man from a single plant genus important for agriculture, horticulture, bioremediation, medicine, soil conditioners, composting crops, and in the production of edible and industrial oils. Many endophytes are horizontally transmitted, but some can colonize the plant's reproductive tissues,

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and this gives these symbionts an efficient mechanism of propagation via plant seed (termed vertical transmission).

Methods. This study surveyed 83 wild and landrace *Brassica* accessions composed of 14 different species with a worldwide distribution for seed-associated bacterial endophytes. Seed was stringently disinfected, sown within sterile tissue culture pots within a sterile environment and incubated. After approximately one-month, direct isolation techniques were used to recover bacterial endophytes from roots and shoots of symptomless plants. -Bacteria were identified based on the PCR amplification of partial 16S rDNA gene sequences and annotated using the BLASTn program against the NCBI rRNA database. A diversity index was used as a quantitative measure to reflect how many different bacterial species there were in the seed-associated microbial community of the *Brassica* accessions sampled.

Results. Bacterial endophytes were recovered from the majority of the *Brassica* accessions screened. *Methylobacterium*, known to stimulate plant development, was the dominant genus composing 56% of the culturable isolated bacterial community and was common in 77% of accessions possessing bacterial endophytes. This is the first report that investigates the seed-associated endophytic microorganisms of wild *Brassica* species.

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Introduction

Endophytes are a diverse sub-group of microorganisms that reside inside the tissues of nearly every vascular plant and, for at least part of their life cycle, do not cause any immediate symptoms (Card et al. 2016; Porras-Alfaro & Bayman 2011; Wilson 1995). However, not all endophytes remain within their plant host throughout their entire life cycle. Additionally, some may change their behavior, from mutualistic to commensalism or even pathogenic, due to a change in the environment, during host senescence or when the host is stressed (Aly et al. 2011; Fisher & Petrini 1992). Endophytes can be found in nearly every type of plant organ, in both vegetative (e.g. leaves, roots and shoots) and reproductive (e.g. flower and seed) tissues (Rodriguez et al. 2009). The presence of bacterial endophytes within the reproductive tissues has been reported for many plant species (Mundt & Hinkle 1976), including coffee (Vega et al. 2005), cotton (Adams & Kloepper 1996), cucumber (Khalaf & Raizada 2016), eucalyptus (Ferreira et al. 2008), oilseed rape (Granér et al. 2003), maize (Rijavec et al. 2007), Norway spruce (Cankar et al. 2005), tobacco (Mastretta et al. 2009) and rice (Elbeltagy et al. 2000; Okunishi et al. 2005). These seed-associated bacterial endophytes may be disseminated from one generation to the next, persisting in the next population of plants (López-López et al. 2010) and is indicative of their ability to vertically transmit. Plant hosts harboring endophytes can gain additional advantageous traits, granting them an ecological advantage over individuals lacking these microorganisms and/or other plant species that occupy a similar ecological niche. These benefits include greater resistance to abiotic and biotic stresses (Hallmann et al. 1997; Mastretta et al. 2006) as well as plant growth promotion (Azevedo et al. 2000).

Modern *Brassica* cultivars were originally domesticated from species mostly originating from Europe (Rakow 2004), although now many *Brassica* crops, particularly *B. napus* (oilseed rape), *B. rapa* (turnip) and *B. oleracea* (cabbage), are extensively cultivated throughout the world. These species are a major source of vegetables for human consumption and for forage, ornamental plants, condiments, medicinal crops, green manure, bioremediation, and as very important sources of edible and industrial oils (Dixon 2007; Gómez-Campo 1980). A wide range of insect pests, such as aphids (*Brevicoryne brassica*), diamond back moth (*Plutella xylostella*) and flea beetles (*Phyllotreta* and *Psylliodes* spp.), in addition to several diseases, such as clubroot (caused by *Plasmodiophora brassicae*), phoma stem canker (caused by *Leptosphaeria maculans*), and sclerotinia stem rot (caused by *Sclerotinia sclerotiorum*) cause extensive damage to *Brassica* crops worldwide (Kimber & McGregor 1995) with few or no control options available (Granér et al. 2003).

Most studies investigating endophytes of *Brassica* have focused on isolating microorganisms from the vegetative tissues of modern-day cultivars (Germida et al. 1998; Narisawa et al. 1998; Sheng et al. 2008; Sunkar & Nachiyar 2013; Zhang et al. 2014). However, this strategy may be restrictive as the diversity and frequency of endophytic species found in domesticated crops is assumed to be much lower than in their respective wild relatives (Mousa et al. 2015; Putra et al. 2015). Additionally, targeting endophytic species that are associated with the reproductive plant tissues (those microorganisms that are seed-borne or seed-transmitted) would greatly aid the marketing of potential commercial products (Card et al. 2016; Card et al. 2015). This study focused on developing a strategy for screening wild and landrace *Brassica* species for mutualistic, seed-associated endophytes that may offer beneficial traits to elite *Brassica* cultivars.

Materials & Methods

Brassica germplasm

Sixty-four accessions (49 wild and 15 landraces) of *Brassica* (*Table S1*), encompassing a diverse number of species, with a worldwide distribution, were obtained from three international genebanks, namely The United States Department of Agriculture (USDA) via The Germplasm Resources Information Network (GRIN), The Leibniz-Institute of Plant Genetics and Crop Plant Research (IPK Gatersleben) and The Nordic Genetic Resource Centre (NordGen). These accessions were imported into the Margot Forde Germplasm Centre (MFGC), New Zealand's national genebank of grassland plants (on seed import permit no. 2015058982). A further 19 populations of wild *Brassica* were collected locally, within the Manawatu-Wanganui region situated in the lower half of the North Island of New Zealand. All 83 accessions were catalogued and stored at 0°C and 30% relative humidity within the MFGC.

Screening for seed-associated bacterial endophytes

Two surface disinfection protocols were developed to remove non-target microorganisms (such as saprophytic microorganisms) associated with the seed surface of the aforementioned *Brassica* accessions. Initially all 83 accessions were surface disinfected using the following protocol: seeds were washed for five min in 5% aqueous Tween-20® solution (Sigma-Aldrich Inc., New Zealand), two min in 70% ethanol, 10 min in 0.5% sodium hypochlorite, one min in 70% ethanol and rinsed three times in sterile water. To assess the efficacy of the surface disinfection protocols, 3 × 20 µL drops of tap water from the last rinse were plated onto nutrient agar (NA), (CM003, Oxoid Ltd., UK). Petri plates containing the NA were incubated for two weeks at 22°C and inspected daily for microbial growth with the aid of a dissecting microscope (Carl Zeiss (N.Z.) Ltd., New Zealand). For those accessions where saprophytic microorganisms were initially observed, a second surface disinfection protocol was applied whereby the same procedure listed above was repeated except for one modification; seeds were immersed for 10 min in 2% sodium hypochlorite rather than a 0.5% solution. Seed were then dried on filter paper (110 mm, Thermo Fisher Scientific Ltd., New Zealand) within a sterile environment and stored at 4 ºC in the dark. To induce germination, seeds were dipped into sterile 0.2% KNO_3 solution and immediately plated onto Petri plates containing 1.5% water agar (WA), with 10 seeds per plate. Petri plates were incubated at 4 ºC in the dark for 72 h to break seed dormancy. Dormant accessions (as tested for 15 accessions) resulted in either zero or poor germination without this process and were subsequently transferred to a custom-built growth chamber at 22-25 ºC and a 16/8 h (light/dark) photoperiod.

Seed and the subsequent seedlings were examined daily under a dissecting microscope and those exhibiting any obvious epiphytic microbial growth were discarded. After 2-3 days, 10 clean seedlings, from each accession, were transferred to sterile tissue culture pots, 98 mm diameter (2105646, Alto Ltd., New Zealand) containing Murashige & Skoog (M&S) basal salts (Murashige & Skoog 1962) with minimal organics (Sigma-Aldrich, New Zealand), plus 3% sucrose and 1.5% agar (Ali et al. 2007). Pots were placed in the growth chamber (with the same settings as described earlier) and visually assessed every day for a month using a dissecting microscope. Plants were discarded if they showed any disease symptoms or any saprophytic microbial growth. Four clean seedlings were finally selected from each accession and subsequently dissected into two components; shoot and root. These organs were further dissected into 2-3 mm^2 pieces using sterile forceps and a scalpel. Ten pieces per organ type from each seedling were transferred to Petri plates containing NA. Petri plates were incubated for three weeks at 22 ºC in the dark and checked daily under a dissecting microscope for microbial growth. Bacterial colonies arising from dissected tissue pieces were selected, sub-cultured and checked for purity. Representative bacterial isolates were then sub-cultured onto fresh NA using a sterile loop and stored in 25% glycerol at -80 °C.

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Identification of seed-associated bacterial endophytes

Bacterial isolates were identified based on the PCR amplification of partial 16S rDNA gene sequences (Weisburg et al. 1991). PCR was performed directly on suspensions of each purified bacterial colony as follows: each colony was suspended in 10 µL Milli-Q® water in a standard 0.2 mL PCR tube (Axygen™, USA) and frozen at -20˚C before being thawed and heated at 65˚C for 30 min. 1 µL of suspension was added to the PCR reaction containing 5 µL 10X PCR buffer, 1.5 µL $MgCl_2$ (50 mM), forward primer, 27F (5`-AGAGTTTGATCCTGGCTCAG,1 µL, 10 µM), reverse primer R1497, (5`- CCTATATCGCCGGTAATT, 1 µL ,10 µM), 0.4 µL dNTPS (25 mM), 0.25 µL Taq-polymerase and 39.85 µL sterile Milli-Q water to make a 50 µL PCR reaction. PCR was performed in a thermocycler (Bio-Rad C1000 Touch™, Bio-Rad Laboratories Inc., USA) with the following conditions: an initial step of 95 ºC for 5min was followed by 36 cycles of 94 ºC for 30 s, 56 ºC for 60 s, 72 ºC for 90 s and a final step of 72 ºC for 10 min; PCR amplification products were confirmed by electrophoresis in a 1.5% agarose gel and purified using the DNA clean & concentrator kit (Zymo Research Corporation, USA) prior to Sanger sequencing (Sanger & Coulson 1975) (New Zealand Genomics Ltd., New Zealand). The raw sequence ab1 files were imported into the software package Geneious Prime® version 2019.1.1 (Biomatters Ltd., New Zealand) and were quality trimmed using an error probability of 0.05. Those sequences with a region of high quality greater or equal than 600 bp were kept and annotated using the BLASTn program against the NCBI rRNA database. The sequences were aligned using the multiple alignment program MAFFT (Katoh & Standley 2013), and a Maximum Likelihood phylogenetic tree was generated using the software Mega X (Kumar et al. 2018) using a general time reversible model and validated by 100 bootstrap cycles. All sequence data were deposited in GenBank under file SUB6483552: MN629046 - MN629135. Simpson`s diversity index (Simpson 1949) was used as a quantitative measure to reflect how many different bacterial species there were in the seed-associated microbial community of the *Brassica* accessions sampled.

Assessing plant growth promotion

Two isolates of *Methylobacterium*, namely *Methylobacterium fujisawaense* (isolate B82) and *Me. phyllosphaerae* (isolate B64), were selected due to their high tissue colonization rate in their original host plants. The bacteria were plated on NA and incubated for two weeks at 22 ºC. For each isolate, cells were scraped from the Petri plates using a loop and transferred to an aqueous Tween-20® solution. Concentrations were adjusted to 10^9 cells per ~~ml~~ mL using a haemocytometer. Oilseed rape, cv. King was selected as the novel host plant. Seeds were surface disinfected, as described earlier, and placed on a filter paper under a laminar flow cabinet to dry. They were then transferred to Petri plates containing 2% ~~water agar (~~WA~~)~~ and incubated at 22 ºC in a custom-made lighting room with 18/6 h~~r~~ (light/dark photoperiod) to initiate germination. The root tip of each seedling was excised with a sterile scalpel, dipped into the prepared bacterial suspension and transplanted into sterile plastic pots (7 × 15 cm) containing autoclaved potting mix (50% fine bark, 12.5% compost and 25% pumice plus nutrient, gypsum and Agri-lime). Control seedlings, after excising the root, were dipped in sterile water containing one drop of

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Tween-20® per ~~litre~~liter. Pots were watered equally, and lids placed on top. Pots were transferred to a plant growth chamber (A1000, Conviron Asia Pacific Pty Ltd., Australia) set at 18 ºC with a 16/8 h~~r~~ (light/dark) photoperiod. The experiment was laid out in a completely randomized design with eight replications. Each experimental unit comprised five pots/plants. After one month, plants were removed, and all soil debris cleaned by washing under a water tap. Each plant was placed on a filter paper for 8 h~~r~~ at room temperature (20-25 ºC) to completely dry. The seedlings were weighed and the mean weight of five plants in each experimental unit were used for analysis of variance (ANOVA) using SPSS software (IBM© SPSS© Statistics, version 24).

Dual culture test

The antifungal activity of a representative isolate of each bacterial species was assessed *in vitro* (dual culture) against the target pathogen, *L. maculans*. The *L. maculans* strain Lm145 utilized was a virulent pathogen of *Brassica* sp., previously isolated from a swede crop in New Zealand (Lob 2014). Bacterial endophytes, previously stored at -80 ºC, were thawed, streaked onto NA and incubated for two weeks at 22 ºC. For each bacterial isolate a cell suspension (10^9 cells per ~~ml~~mL) was prepared and 50 µL streaked in a straight line across the center of a Petri plate. Control treatments were streaked with only sterile water mixed with one drop of Tween-20® per ~~litre~~liter. Petri plates were incubated for two weeks at 22 ºC and then two mycelial plugs (5 mm diameter) were taken from the actively growing region of a two-week old *L. maculans* culture and placed 25 mm from the edge of each Petri plate opposite each other; there were 10 replicate plates for each treatment. When the two fungal colonies placed on opposite sides had grown sufficiently to close the gap between them, the distance between the bacterial colony and the *L. maculans* colony was measured using a digital caliper (Mitutoyo Corporation, Japan). The inhibition zone was rated using a 5 point scaling system~~;~~: 4 (very high), inhibition zone >10 mm; 3 (high), imbibition zone 5-10 mm; 2 (medium), inhibition zone <5 mm; 1 (low), *L. maculans* growth stopped at the bacterial streak line; 0 (zero), no inhibition zone with *L. maculans* typically growing over the bacterial streak (Hammoudi et al. 2012).

Results

Seed-associated bacterial isolates

In total, 54 accessions (44 wild and 10 landrace), out of the total 83 accessions surface disinfected and sown, resulted in symptomless plants when germinated and subsequently grown on M~~&~~S medium. The remaining 29 accessions all exhibited epiphytic fungal growth, with most colonies identified as *Alternaria* sp. These plants were all destroyed by autoclaving. After incubation, bacterial colonies were detected from 48 symptomless wild *Brassica* accessions (38 wild and 10 landrace). Six accessions did not result in any bacteria being isolated.

Identification of seed-associated bacteria

A sample of 90 bacterial isolates, representing the morphological diversity of all the observed bacterial colonies that developed from dissected *Brassica* tissue, were sequenced to determine

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their species identity. 16S rDNA gene sequencing identified 19 different bacterial species belonging to three phyla, namely Actinobacteria, Firmicutes and Proteobacteria (*Fig. 1 and Table 1*). According to the phylogenetic tree (Fig. 1) the most frequently isolated species were *Methylobacterium fujisawaense* (50 isolates), *Stenotrophomonas rhizophila* (10 isolates) and *Pseudomonas lactis* (9 isolates). Three species were identified from the Actinobacteria, namely *Kocuria palustris*, *Micrococcus aloeverae*, and *Plantibacter flavus* while two species were identified from the Firmicutes phylum, namely *Bacillus mycoides* and *Paenibacillus hordei*. Simpson's diversity index recorded a value of 0.74 indicating that these wild and landrace *Brassica* accessions contained a high diversity of seed-associated bacteria. Nevertheless, *Methylobacterium* spp., predominantly *Me. fujisawaense* and the closely related species *Me. phyllosphaerae*, *Me. oryza* and *Methylorubrum extorquens* constituted 56% of the bacteria isolated and were common in 77% of the *Brassica* accessions screened (38 ~~accessions;~~accessions: 28 wild and 10 landrace). These were also distributed among multiple *Brassica* species including *Bra. barrelieri*, *Bra. elongate, Bra. gravinae*, *Bra. indica, Bra. juncea*, *Bra. napus*, *Bra. nigra* and *Bra. rapa* sourced from five continents, namely Africa, Asia, Australasia, Europe and North America.

Plant growth promotion

Oilseed rape plants that were inoculated with two isolates of *Methylobacterium* showed a significant ($P<0.01$) increase in their dry weight when compared to the un-inoculated control plants (Fig. 2). Plants inoculated with *Me. fujisawaense* (B82) had a mean weight of 1.33 g/plant, while those inoculated with *Me. phyllosphaerae* (B62) displayed a mean weight of 0.88 g/plant compared to a mean weight of 0.69 g/plant for the uninoculated control plants (Fig. 2).

Dual culture test

The results from the dual culture assays indicate that some of the bacterial isolates showed antagonistic behavior towards *L. maculans*. The strongest inhibition effect was observed with isolates of *Me. fujisawaense* and *Me. phyllosphaerae*, *N. resinovorum*, *Ps. lactis*, *Pl. flavus* and *St. rhizophila* that all showed a high, clear inhibition zone between the edge of the bacterial streak and the pathogen. However, *L. maculans* was not affected by *K. palustris*, *Sphingom. yantingensis*, *Sphingom. insulae*, *Ba. mycoides* or *Brevundimonas vesicularis*; with no inhibition zone produced.

Discussion

This study investigated the cultivable bacterial community persisting in wild and landrace *Brassica* seed. ~~Seventeen~~ Nineteen bacterial species were isolated from 83 accessions, belonging to eight *Brassica* species, covering five continents, with some of the accessions more than 20 years old. The bacterial genera to which these species belong have been previously reported in the literature as seed endophytes of a diverse number of plant species. For example, *Methylobacterium* and *Paenibacillus* spp. have both been described as seed endophytes from

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Eucalyptus (Ferreira et al. 2008), *Oryza sativa* (Mano et al. 2006) and *Phaseolus vulgaris* (López-López et al. 2010), while *Bacillus* and *Micrococcus* spp. are common seed endophytes of *Coffea arabica* (Vega et al. 2005) and *O. sativa* (Mano et al. 2006). Our results indicate that the diversity of bacterial endophytes in seed of wild *Brassica* is relatively high with most of the bacterial species identified belonging to the Proteobacteria, the major phylum of gram-negative bacteria. This is consistent with earlier work that showed the seed microbiome of oilseed rape were colonized mostly by Proteobacteria and that individual cultivars each had their own unique microbiome profile (Rybakova et al. 2017).

Development of an effective surface disinfection protocol was paramount to this study. A protocol that was too harsh could sterilize the seed and kill any potentially beneficial microorganisms residing in the seed tissues, as well as potentially damaging the seed itself, while a protocol that was too moderate could yield unwanted saprophytic microorganisms residing on the surface of the seed coat. These non-target saprophytes have the potential to outgrow any slower growing endophytic organisms that may be beneficial. Many of these non-target species can colonize the interior tissues of the germinating plant during the emergence of the radicle (Bent & Chanway 2002). The surface disinfection protocol used in this study was not designed to eliminate all organisms living on the seed surface, just to reduce their frequency. For example, *Alternaria* sp., commonly associated with seed coats or pericarps of seed (Harman 1983; Neergaard 2011) was frequently isolated.

Many studies have reported that strains belonging to the same genera identified in our study confer several beneficial traits to their host plants, including enhanced resistance against certain plant pathogens and/or growth promotion (Araújo et al. 2002; Berg & Hallmann 2006; Khan et al. 2014; Rashid et al. 2012; Rout & Chrzanowski 2009; Sessitsch et al. 2004; Ying et al. 2016). We assessed the antagonistic activity of selected isolates of bacterial species against *L. maculans* (the causal agents of phoma stem canker in oilseed rape) through dual culture bioassays and observed that *Me. fujisawaense* and *Me. phyllosphaerae* possessed antagonistic potential against the pathogen. The genus *Methylobacterium* is composed of pink-pigmented facultative methylotrophs (PPFMs) (Dourado et al. 2015) that are able to form endophytic associations with a range of plant species including citrus (Araújo et al. 2002), cotton (Madhaiyan et al. 2012), eucalyptus (Andreote et al. 2009), mangrove (Dourado et al. 2012), peanut (Madhaiyan et al. 2006b), pine (Pohjanen et al. 2014) and tobacco (Andreote et al. 2006). PPFMs are not pathogenic to their plant hosts (Idris *et al.* 2006) making them ideal candidates for endophytic biological control strategies (Omer *et al.* 2004). Additionally, *Methylobacterium* spp. are able to enhance plant growth through several mechanisms, including, nitrogen fixation (Lee et al. 2006; Menna et al. 2006; Sy et al. 2001), phytohormone production such as cytokinins and auxins (Madhaiyan et al. 2006a; Meena et al. 2012; Trotsenko et al. 2001), interact with and inhibit plant pathogens (Araújo et al. 2002; Lacava et al. 2004; Poorniammal et al. 2009), promote plant growth (Madhaiyan et al. 2006a; Madhaiyan et al. 2006b; Tani et al. 2012), induce higher

photosynthetic activity (Cervantes-Martínez et al. 2004), induce systemic resistance (Madhaiyan et al. 2006b), decrease environmental stress (Muller et al. 2011) and immobilize heavy metals (Dourado et al. 2012). We analyzed the fresh weight of seedlings of an oilseed rape cultivar under growth chamber condition when the roots were inoculated with two isolates of *Me. fujisawaense* and *Me. phyllosphaerae* and found that inoculated plants had a higher growth rate that non-inoculated plants. Cultivated *Brassica* crops such as oilseed rape, have a high nitrogen demand (Rathke et al. 2006) and their cultivation is reliant on fertilization with nitrogen rich products. These crops usually have low nitrogen use efficiency and this is a specific target for the breeding of new cultivars (Bouchet et al. 2016; Bouchet et al. 2014). The frequent presence of *Methylobacterium* in wild *Brassica* species, that are usually found within infertile soils, such as those where some of the wild species used in our study were collected suggests that this symbiosis improves the development of the host plant. These bacteria may therefore possess traits for use as plant growth promoters in artificial *Brassica* hosts such as domesticated cultivars.

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This study isolated species of *Methylobacterium*, and the closely related *Methylorubrum extorquens* (Green & Ardley 2018), from above and below ground plant organs (shoot and root, respectively). As morphologically similar isolates were identified from multiple root and shoot tissue pieces belonging to the same individual plant, we speculate that these bacterial isolates are capable of systemic plant colonization. Additionally, these tissues were dissected from symptomless seedlings grown from surface disinfected seed under sterile conditions and therefore this strongly suggests that these bacteria are vertically transmitted. The *Methylobacterium* species isolated in this study were present in a range of plant accessions originating from a geographically diverse set of countries with varied altitude. This is consistent with other reports of endophytic microbes, for example, among *Zea* spp. which were found across species grown in wide range of geographical locations (Johnston-Monje & Raizada 2011).

It has been reported the age of seed may considerably influence the seed microbiome (Cankar et al. 2005). Indeed, no bacteria were isolated from six accessions that had been stored for more than 15 years. However, one accession that was over 26 years old gave rise to *Methylobacterium* indicating that this bacterium can adapt and survive in seed tissues for a long period of storage time. Mano *et al.* (2006) reported that only certain bacteria such as *Methylobacterium* are able to reside inside rice seed. These endophytic bacteria enter the seeds during the seed maturation stages and are tolerant to high osmotic pressure. The isolates possess a high degree of amylase activity, which may aid survival in the seed (Mano et al. 2006).

Conclusions

Although three species of *Methylobacterium*, namely *Me. extorquens*, *Me. mesophilicum* and *Me. goesingense*, were previously identified in *Thlaspi goesingense* belonging to the wider *Brassicaceae* family (Idris et al. 2006), to our knowledge this is the first report to describe the

isolation and identification of endophytic bacteria of seeds of wild and landrace *Brassica* species. We present a straight-forward strategy to screen and cultivate seed-associated endophytes with possible beneficial traits. Focusing our efforts on seed-associated organisms may facilitate novel endophyte technologies that could be incorporated into future crop seed (Berg et al. 2017). This approach would then also be advantageous to companies that wish to invest in the commercialization of such products as they can lower their financial risk in terms of delivering a suitable efficacious product to farmers whilst protecting their IP. The latter is possible because an elite plant cultivar and the biological control agent can be protected together in one commercial seed product entity. This means of propagation relies on the plant's reproductive strategy and may aid the marketing of any potential plant-endophyte product (Card et al. 2016).

Acknowledgements

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