Out of the Ground: Aerial and Exotic Habitats of the Melioidosis Bacterium *Burkholderia pseudomallei* in Grasses in Australia

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Summary

Melioidosis is an emerging infectious disease of humans and animals in the tropics caused by the soil bacterium *Burkholderia pseudomallei*. Despite high fatality rates, the ecology of *B. pseudomallei* remains unclear. We used a combination of field and laboratory studies to investigate *B. pseudomallei* colonization of native and exotic grasses in northern Australia. Multivariable and spatial analyses were performed to determine significant predictors for *B. pseudomallei* occurrence in plants and soil collected longitudinally from field sites. In plant inoculation experiments, the impact of *B. pseudomallei* upon these grasses was studied and the bacterial load semi-quantified. Fluorescence-*in-situ*-hybridization and confocal laser-scanning microscopy were performed to localize the bacteria in plants. *B. pseudomallei* was found to inhabit not only the rhizosphere and roots but also aerial parts of specific grasses. This raises questions about the potential spread of *B. pseudomallei* by grazing animals whose droppings were found to be positive for these bacteria.

In particular, *B. pseudomallei* readily colonized exotic grasses introduced to Australia for pasture. The ongoing spread of these introduced grasses creates new habitats suitable for *B. pseudomallei* survival and may be an important factor in the evolving epidemiology of melioidosis seen both in northern Australia and elsewhere globally.

Introduction

Melioidosis is an infectious disease affecting humans and animals in the tropics. It is caused by the Gram-negative environmental β-Proteobacterium *Burkholderia pseudomallei*. Melioidosis is a major cause of community-acquired pneumonia and septicemia in endemic areas such as northern Australia and Southeast Asia, with mortality still over 50% in some locations (Cheng *et al.*, 2005; White, 2003). Melioidosis is also seen as an emerging public health threat, with both rising incidence rates in endemic areas (Currie *et al.*, 2010;

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Limmathurotsakul *et al.*, 2010a) and cases increasingly being described from locations not previously considered to be endemic (Currie *et al.*, 2008). With the high mortality from melioidosis, the potential for airborne transmission of *B. pseudomallei* and its intrinsic resistance to antibiotics commonly used for sepsis, *B. pseudomallei* has been classified as a Category B biothreat agent by the US Centers for Disease Control (Rotz *et al.*, 2002).

Since the sequencing of the first genome of *B. pseudomallei* (Holden *et al.*, 2004), there have been numerous publications analyzing potential pathogenicity determinants of *B. pseudomallei* (French *et al.*, 2011; Galyov *et al.*, 2010). Nevertheless, the search for why *B. pseudomallei* can be such a dangerous pathogen has not been matched by studies defining the ecology of *B. pseudomallei* and many uncertainties remain about its global distribution (Currie *et al.*, 2008) and its habitat in endemic locations. Defining the environmental determinants of *B. pseudomallei* presence, persistence and propagation is critical to understanding and potentially controlling the local and global spread of *B. pseudomallei* that is thought to be occurring.

B. pseudomallei is known to prefer moist, slightly acidic, nutrient-rich soil (Inglis *et al.*, 2006; Kaestli *et al.*, 2009; Palasatien *et al.*, 2008; Wuthiekanun *et al.*, 1995) and also occurs in water (Draper *et al.*, 2010; Inglis *et al.*, 2000; Mayo *et al.*, 2011). In a survey on *B. pseudomallei* occurrence in the Darwin region (12°S latitude) of northern Australia, we found a significant association of these bacteria with grass- and roots-dense areas (Kaestli *et al.*, 2009). This comes as no surprise as bacteria of the genus *Burkholderia* commonly colonize plants and, in particular, the rhizosphere that is the nutrient-rich zone surrounding the roots (Compant *et al.*, 2008; Fiore *et al.*, 2001). Despite the well-known occurrence of *B. pseudomallei* in rice fields (Wuthiekanun *et al.*, 1995), to date, only a few publications have reported a direct association of *B. pseudomallei* with plants: *Burkholderia pseudomallei* was retrieved from the rhizosphere of a wattle shrub (*Acacia colei*) in northwest Australia (Inglis *et al.*, 2000) and Levy *et al.* reported the ability of *B. pseudomallei* to colonize root-associated mycorrhizal fungi (Levy *et al.*, 2003). In laboratory experiments, *B. pseudomallei* was found to have phyto-pathogenic traits with infection of tomato plants and intracellular replication (Lee *et al.*, 2010).

The aim of this study was to investigate further our findings of the association of *B. pseudomallei* with grasses. Grass species found to support the growth of *B. pseudomallei* might serve as bio-indicators for potential presence of *B. pseudomallei* and thus, assist in predicting its distribution and spread both locally and globally. Furthermore, the finding of a close interaction of *B. pseudomallei* with eukaryotic hosts such as plants might have implications for potential future studies on the mechanisms involved in the colonization of other eukaryotic cells by *B. pseudomallei* and specifically its ability to invade and persist in animal and human tissues.

Results

We investigated the association of *B. pseudomallei* with various grasses occurring at known hotspots for *B. pseudomallei* in the Darwin region using a combination of observational longitudinal field studies and laboratory-based grass inoculation experiments. We studied the impact of *B. pseudomallei* upon these grasses, semi-quantified its load and visualized the colonization of *B. pseudomallei* in the rhizosphere, roots and aerial parts using fluorescence *in situ* hybridization (F*IS*H) and confocal laser-scanning microscopy.

B. pseudomallei occurs significantly more often in areas with exotic grasses

In the Darwin region, four sites next to streams were chosen to study the occurrence of *B. pseudomallei* in areas dense in grasses commonly occurring in the Darwin region. These

sites had previously been positive for *B. pseudomallei* (Kaestli *et al.*, 2009). Native grass species (including *Sorghum* spp. and *Whiteochloa* spp.) were present at each study site. Exotic grasses were found at three of these four sites, such as *Brachiaria humidicola* cv Tully (Tully Grass), *Pennisetum pedicellatum* and *polystachion* (Mission Grass) and *Paspalum plicatulum* (Paspalum).

Using a grid-like sampling approach over two consecutive dry and wet seasons, soil and grasses of these sites were repeatedly screened for presence of *B. pseudomallei* by an enriched soil/plant DNA extraction method and TTS1 real-time PCR (Kaestli *et al.*, 2007). We found 37% (142/380) of soil samples, 18% (38/217) of collected grass roots and 23% (31/135) of foliage samples positive for *B. pseudomallei* at these sites (see Table 1). A significant difference was evident in *B. pseudomallei* load in grass foliage between the dry and wet season, with a higher proportion of leaves being *B. pseudomallei* positive in the dry (42% of screened leaves (13/31)) as opposed to the wet season (17% (18/104), Fisher's Exact, P=0.007).

There was strong evidence that more root and foliage samples of exotic grasses were *B. pseudomallei* positive (47% of screened exotic grasses; 43/91) than native grasses (10%; 13/126)(Fisher's Exact, P<0.001). Soil associated with native grasses (mostly annuals) showed a higher *B. pseudomallei* detection rate in the wet (32% (34/107)) as opposed to the dry season (20% (31/157), Fisher's Exact, P=0.030). No such trend was evident for the soil associated with exotic grasses (mostly perennials) with year-round high *B. pseudomallei* prevalence, 71% (40/56) in the dry and 62% (37/60) in the wet season.

A multivariable logistic regression analysis clustered for sites was performed to identify environmental factors significantly contributing to the occurrence of *B. pseudomallei* in soil at these sites (see Table 2). For the dry season, exotic grasses were the most dominant predictor for presence of *B. pseudomallei* followed by moist to wet soil and a less acidic soil pH (pH at *B. pseudomallei* positive sites in the dry season: 95 % confidence interval 5.1–5.6; at negative sites 5.0–5.3). In the wet season, these sites were partially inundated and significant predictors for presence of *B. pseudomallei* in the soil were road drainage, exotic grasses and the soil colours reddish grey and reddish yellow. Reddish grey and yellow soil colours have previously been associated with the presence of *B. pseudomallei* (Kaestli *et al.*, 2007; Thomas *et al.*, 1979). These soil colours are commonly found in the Top End of Australia, often indicating imperfect drainage and oxidized iron. Indeed, this was also confirmed in our study by the redoxic hydrosol soil profiles of these sites with a ferricrete layer at site E (see Supplement Table 1). Sandy clay loam topsoil of site E was deposited from upland red massive earths which have formed under alternating tropical wet and dry conditions with weathering and leaching of mainly siliceous parent materials and segregation of iron and manganese oxides (Stace *et al.*, 2011). Topsoil (n=6) of site E also had a high reactive iron content with a mean above 3000 mg/kg (see Supplement Table 2).

We found the occurrence of *B. pseudomallei* in soil to be clustered at sites with exotic grasses; at site E there was a significant positive spatial autocorrelation in the 0–0.0005 degree (0–50 meters) band (Geary's c 0.67–0.88, P<0.001) which matched the patchy distribution of Tully Grass which also showed a significant positive spatial correlation (Geary's c 0.17–0.65, P<0.001). This clustering was also visualized using indicator kriging to map Tully Grass as well as soil moisture against *B. pseudomallei* positive and negative holes (see Figure 1).

B. pseudomallei culture isolates from these field sites were subject to molecular typing, namely multilocus sequence typing (MLST) (Godoy *et al.*, 2003). Sequence types (STs)

commonly retrieved had previously been isolated from clinical cases and environmental sources in rural Darwin (e.g. ST 109, 144, 266).

FISH/CLSM and real-time PCR studies show colonization of B. pseudomallei of roots and aerial parts of grasses

F*IS*H and confocal laser-scanning microscopy (CLSM) analysis were performed to determine the colonization behavior of *B. pseudomallei* in roots and foliage of grasses collected from the field sites as well as from inoculated plant cultures (see Figure 2). All roots and foliage positive for *B. pseudomallei* by F*IS*H analysis were also found to be positive for *B. pseudomallei* by *B. pseudomallei* specific TTS1 real-time PCR (Kaestli *et al.*, 2007). Microscopy showed that *B. pseudomallei* cells were smaller in size in grass samples when compared to *B. pseudomallei* in culture, with an approximate size of 1–2 μm. *B. pseudomallei* was detected in the rhizosphere of all grass species examined (Tully, Paspalum, Mission Grass collected from field sites as well as inoculated *Sorghum intrans* and *Oryza rufipogon* (Wild Rice)). While the bacteria often clustered, they were mainly found around root hairs and in junctions of root branches. Less frequently, *B. pseudomallei* was also detected inside roots such as of Mission or Tully Grass from field sites, i.e. inside root hairs and root cortex cells as well as in between the cortex cells. A small number of *B. pseudomallei* were found in the xylem of a field collected Paspalum from field site B. In the foliage of grasses (namely Tully Grass, Paspalum and Mission Grass from field sites), *B. pseudomallei* were found around the stomata, at the surface of these openings as well as within the stomatal guard cells. In Wild Rice where the rhizosphere was inoculated with *B. pseudomallei*, the bacterium was detected along the vascular bundles of the foliage, at the surface of the lower side of the leaf in a gully-like structure formed by the vascular bundles.

B. pseudomallei colonization exerts a pathogenic effect upon exotic Mission Grass in contrast to native Wild Rice

140 seedlings of Mission Grass, Tully Grass and Wild Rice were inoculated with no, low or a high dose of *B. pseudomallei* (see Supplement Figure 1). After four weeks, there was evidence that *B. pseudomallei* had a pathogenic effect upon Mission Grass. Seedling mortality increased proportionally from the negative to the low and high dose inoculation group (4% vs. 21% vs. 37% dead seedlings; n=62; Fisher's Exact, P=0.033). Furthermore, a high inoculation dose was a predictor for death of Mission Grass seedlings with an unadjusted Odds Ratio of 13.4 (95% CI 1.5–122.1, P=0.021). There was also a trend of reduced foliage in Mission Grass seedlings which were inoculated at a high dose and alive after 4 week; however, this trend was not statistically significant and may reflect low numbers (median growth 23.5 cm in negative and low dose, 95% CI 13–38 (n=38) as opposed to 4.5 cm, 95% CI 0–42.7 cm for high dose (n=12), Mann-Whitney, P=0.16). Although not statistically significant, a higher proportion of dead Tully Grass was observed in the high inoculation group as compared to the low inoculation and negative groups (27% dead plants (4/15) vs 9% (3/32), P=0.188) and a decreased leaf growth was found (see Supplement Figure 1).

No decrease in foliage or root growth was evident in each of the three grass species when inoculated with a low amount of *B. pseudomallei*.

B. pseudomallei did not exert any obvious negative effects on Wild Rice. There was no reduction in foliage, root growth or seedling mortality upon increased dose of inoculation. Interestingly, there was evidence for increased root growth in the high inoculation dose (median growth 4 cm in negative and low dose, 95% CI 3.4–7.6 (n=13) as opposed to 8 cm, 95% CI 6.3–9.7 cm for high dose (n=7), Mann-Whitney, P=0.038).

Semi-quantitative analysis of Burkholderia and B. pseudomallei load in grasses

Semi-quantitative analysis of *B. pseudomallei* load was carried out in 90 root and aerial samples of plants from the above plant growth and time series experiments (see Figure 3 and Supplement Figure 2) restricting the analysis to those plants which were inoculated at least three days prior to harvesting. There was evidence for the median *B. pseudomallei* load being more than 100 times higher in Mission Grass roots as compared to Wild Rice roots (bootstrap estimate 95% CI 18–205, Mann-Whitney Test, P=0.009)(see Figure 3). A positive correlation was found between *B. pseudomallei* counts in roots and leaves (Spearman's rho 0.44, P=0.008).

A comparison of semi-quantitative load of bacteria of the genus *Burkholderia* in grasses of the plant growth experiment revealed evidence for 19 times less *Burkholderia* in Mission Grass leaves than in leaves of Tully Grass (median bootstrap estimate 95% CI 1.3–37.6, Mann-Whitney Test, P=0.010) or 7 times less than in leaves of Wild Rice (95% CI −16.8–31.8, P=0.055) though the latter was not statistically significant.

Discussion

To date, the literature has mainly suggested soil and surface water as the natural habitat of *B. pseudomallei* (Cheng *et al.*, 2005). To our knowledge, only two reports showed an association with the rhizosphere of plants (Inglis *et al.*, 2000; Levy *et al.*, 2003). Using real-time PCR and F*IS*H, we found *B. pseudomallei* not only in the rhizosphere of various grasses in the Top End of Australia but also endophytically, colonizing both roots and aerial parts.

The rhizosphere and particularly areas around root hairs and lateral root emergence are rich in root exudates and nutrients and an ideal habitat for a saprophytic bacterium such as *B. pseudomallei*. This prime ecological niche was described as a "playground and battlefield" for microbes (Raaijmakers *et al.*, 2009). The fierce competition among the microbial community results in selection pressure for developing traits such as improved iron scavenging by siderophore production (Alice *et al.*, 2006; Lemanceau *et al.*, 2009), exhibiting antagonistic activities against other microbes and optimizing survival mechanisms in the eukaryotic host, be it a plant or root-associated mycorrhizal fungi (Berg *et al.*, 2005; Levy *et al.*, 2003; Raaijmakers *et al.*, 2009). Some of these traits are likely to also aid in successful colonization of human tissues and organs and thus, the rhizosphere is a known source of versatile human opportunistic pathogens such as bacteria of the *B. cepacia* complex (Berg *et al.*, 2005; Fiore *et al.*, 2001). Other bacteria with human-pathogenic traits and previously associated with plants include common vegetable contaminants such as bacteria of the Enterobacteriaceae family, *Listeria* spp. and *Pseudomonas* spp. (Schwaiger *et al.*, 2011)

F*IS*H microscopy also suggested the presence of *B. pseudomallei* inside and in between root cortex cells. Entry of *B. pseudomallei* into roots might be similar to that of some rhizobial bacteria and occur via natural openings or wounds in the epidermis such as caused by lateral root emergence (Roberts K ed., 2007). A few *B. pseudomallei* were also found in the xylem, which transports dissolved mineral nutrients and water from the roots into the aerial parts of the plant.

We detected various *B. pseudomallei* at the surface of and inside stomata and veins of leaves. It is unclear whether the bacteria invaded the leaf internal tissue via roots, xylem and vascular bundles and/or via stomata after leaf surface contamination by *B. pseudomallei* contaminated water droplets from wind/rain, or, in the plant culture experiments by condensation in the culture container. Stomatal openings promote gas exchange and

transpiration. They are a common entry point for bacteria into leaves and some plants have developed molecular defense mechanisms such as bacterial-signal induced stomatal closure (Melotto *et al.*, 2008). Reduced stomatal size has also been associated with resistance to disease (Riikonen *et al.*, 2008). We indeed found the stomata of more resistant Wild Rice to be smaller than the ones of Mission Grass. However, this needs formal study.

Leaves of perennial grasses might also serve as a "refuge" for *B. pseudomallei* in the dry season when surface soil moisture is commonly too low for growth. We found a significantly higher proportion of leaves to be positive for *B. pseudomallei* in the dry as opposed to the wet season. Stomata provide a protected hydrophilic environment and bacteria of the genus *Burkholderia* have previously been found inside stomatal chambers (Compant *et al.*, 2008). We also detected *B. pseudomallei* in natural pockets along vascular leaf bundles which are known to be a protected niche for bacterial epiphytic survival (Melotto *et al.*, 2008).

The fact that *B. pseudomallei* colonizes aerial parts of grasses raises the question whether this also facilitates spread of these bacteria by animals traversing grass fields and/or by oral-fecal transmission by animals such as grazing macropods which feed on grasses. Mission Grass is a preferred diet of wallabies (Phil Hickey, pers. comm.) and we have previously found a significant association of *B. pseudomallei* occurrence with sites containing animal waste including of macropods (Kaestli *et al.*, 2009) and we have detected *B. pseudomallei* in macropod droppings in rural Darwin by molecular detection as described in (Kaestli *et al.*, 2007).

In a survey on *B. pseudomallei* occurrence in rural Darwin (Kaestli *et al.*, 2009), we found that anthropogenic manipulations of the environment such as irrigation or keeping animals have an impact upon *B. pseudomallei* occurrence. We also observed that some areas with seemingly less signs of anthropogenic environmental disturbance but with repeatedly high counts of *B. pseudomallei* showed a dense grass growth, even in the dry season. Work described in this report provides evidence that the major contributor to the high *B. pseudomallei* load at these less disturbed sites are introduced grass species. Given that much of northern Australia is free from other anthropogenic environmental disturbances such as construction and farm animals, the magnitude of contribution to overall *B. pseudomallei* load from imported grasses in these sites is likely to be substantial. These grasses were introduced to Australia in the 20th century as pasture grass, including Tully Grass, Mission Grass, both native to Africa and Paspalum, native to the Americas. Melioidosis case reports from these continents are rare (Currie *et al.*, 2008) and *B. pseudomallei* might only occur in localized foci in these regions. Upon introduction of these grasses to Australia, they have since spread to non-targeted areas and Mission Grass has been declared a weed (Walden *et al.*, 2008). We chose four field sites with a varying degree of exotic grasses and using a grid-like sampling approach (Limmathurotsakul *et al.*, 2010b) and multivariable analysis, exotic grasses were indeed the most dominant predictor for presence of *B. pseudomallei* in the dry season. Invasive plants are known to change the microbial community of the soil and rhizosphere (Batten *et al.*, 2006; Kourtev *et al.*, 2002) such as by plant exudates or by impacting upon the soil composition through changing soil biomass, nutrient status, pH or moisture (Ehrenfeld *et al.*, 2001). Various negative or positive feedback loops between invasive plants and soil microbiota have been shown (Callaway *et al.*, 2004; Reinhart *et al.*, 2006). Invasive grass species in Northern Australia increase biomass and persistence of annual soil wetting (Wurm *et al.*, 2006) and impact upon soil nitrogen cycles (Rossiter *et al.*, 2006) and thus, might also indirectly influence the occurrence of *B. pseudomallei*. Moreover, in contrast to many native grasses which only grow during the wet season, the perennial nature of Paspalum, Tully and Mission Grass allows *B. pseudomallei* a year-

round, stable habitat which results in a high prevalence of these bacteria in the dry season when *B. pseudomallei* occurrence is otherwise usually low (Thomas *et al.*, 1979).

Differences in plant immunity as well as microbial competition between established native and introduced plants might also influence the level of colonization success by *B. pseudomallei*. In plant inoculation experiments using molecular semi-quantification, we found that Wild Rice which is native to melioidosis-endemic northern Australia harbored less *B. pseudomallei* per cm leaf or root compared with Mission Grass. The opposite was evident when comparing the load of bacteria of the whole genus *Burkholderia,* with more *Burkholderia* detected in Wild Rice than in Mission Grass. This was also evident when culturing bacteria from these plants using selective Ashdown's media. The most dominant species cultured from Mission Grass was *B. pseudomallei* while primarily other bacteria such as those of the *B. cepacia* complex were recovered from Wild Rice.

Moreover, we consistently found *B. pseudomallei* recovered from Mission Grass roots or leaves to be of common rough colony morphology type I (Chantratita *et al.*, 2007) which corresponded to the inoculation type. This was in stark contrast to Wild Rice, from which *B. pseudomallei* was mainly retrieved as smooth colony morphology type III. This smooth type was previously associated with an environmental stress response and increased flagella expression which was suggested to assist the bacteria in moving towards more favorable conditions (Chantratita *et al.*, 2007; Tandhavanant *et al.*, 2010).

These observations suggest that *B. pseudomallei* encounters a reduced level of microbial competition and likely, reduced specific plant defense mechanisms in exotic Mission Grass as opposed to in native Wild Rice. Our findings of increased resistance of Wild Rice to *B. pseudomallei* colonization match results by Lee *et al.* (Lee *et al.*, 2010) who showed that rice plants were resistant to *B. pseudomallei* infection. *B. pseudomallei* is now thought to have originated within Australia (Pearson *et al.*, 2009) and Wild Rice and *B. pseudomallei* possibly shared the same habitat for many millennia, suggesting co-evolution resulting in balanced bacterial-host interactions after the likely development of some plant immunity against these opportunistic pathogens.

While high loads of *B. pseudomallei* exerted a pathogenic effect upon Tully or Mission Grass in plant inoculation experiments, plants of these species showed no signs of disease *in situ* at the field sites despite being colonized by *B. pseudomallei*. In native wild rice, there was even evidence for increased root growth upon high dose inoculation of *B. pseudomallei* in plant inoculation experiments. The ecological role of *B. pseudomallei* in plants needs more formal study; we did not detect noteworthy production of indole derivatives such as auxins by *B. pseudomallei* and neither any cellulose degradation (see supplement). While auxin is a plant hormone often produced by plant associated bacteria and found in both, plant growth promoting bacteria and plant pathogens (Patten *et al.*, 1996), the ability to degrade cellulose is common to plant pathogens (Compant *et al.*, 2008).

More studies are needed to explore future implications of our findings of a preferential colonisation of some introduced grass species by *B. pseudomallei* in a melioidosis endemic area. As these grass species are now commonly found in rural areas in Northern Australia, an increased risk of exposure to *B. pseudomallei* for the rural population is possible as a consequence of this spreading. In contrast, while it is to date unknown what interactions occur between *B. pseudomallei* and wild rice *in situ*, due to the main distributions of wild rice being in remote wetlands, such interactions do not currently directly impact upon the risk of exposure to the human population. In terms of global implications and following potential dispersal of *B. pseudomallei* to a non-endemic area, *B. pseudomallei* survival in a new environment not only depends on local climate, physico-chemical soil conditions, soil

disturbance and type of vegetation but as indicated by antibiosis studies (Lin *et al.*, 2011; Marshall *et al.*, 2010) and our data, also on the level of competition from the local established microbial community. The ongoing worldwide spread and use of these grasses for pasture might be contributing to global dispersal of *B. pseudomallei*. Nevertheless, although these grass species now occur worldwide it remains to be seen whether the association with *B. pseudomallei* found in northern Australia is also evident in other parts of the world. The confirmation of colonization by *B. pseudomallei* of invasive grasses beyond Australia would assist in predicting the distribution of *B. pseudomallei* in the environment globally.

To our knowledge, this is the first report on the widespread occurrence of *B. pseudomallei* in grasses in a melioidosis endemic area. Our data indicate that *B. pseudomallei* readily colonizes the rhizosphere of various grasses and is also an endophyte – likely of opportunistic nature - profiting from a protected, hydrophilic and nutrient-rich environment. A habitat in aerial parts of grasses also raises questions about potential dispersal mechanisms involving grazing animals. The ongoing spread of some invasive grass species both within Australia and globally contributes to changes in landscape ecology with new habitats suitable for *B. pseudomallei* survival, with public health implications from exposure of populations to this potentially deadly bacterium.

Experimental Procedures

Field studies and description of field sites and soils

Four field study sites were chosen in rural Darwin (12°S) in the tropical Top End of the Northern Territory of Australia. This location has the highest reported incidence of melioidosis in the world (Currie *et al.*, 2010; Limmathurotsakul *et al.*, 2010a). These field sites were previously found to be repeatedly positive for *B. pseudomallei* and were in riparian zones with a stream marking one side, a drain of a road another side and open woodland and open forest the other two sides. The vegetation of the sites consisted of an open forest of Melaleuca (Paperbarks), Eucalyptus and Pandanus species with a grass understory of native (such as *Sorghum* spp. or *Whiteochloa* spp) and at three sites, introduced grasses (mainly *Brachiaria humidicola* cv Tully (Tully Grass), *Pennisetum pedicellatum* and *polystachion* (Mission Grass) and *Paspalum plicatulum* (Paspalum)). Following the "Australian Soil and Land Survey" Field Handbook (McDonald *et al.*, 1998), soil profiles were classified as redoxic Hydrosols for sites B, P and S and as Anthroposols (in a Redoxic Hydrosol environment) for site E as according to the Australian Soil Classification (Isbell, 2002)(see Supplement Table 1). The topsoil of these sites was generally consistent both within and between sites and consisted of sandy loam to sandy clay, with the predominance of sandy loam followed by sandy clay loam. Sites B and E also had a layer of peat indicating decomposition of plant remains but the organic carbon content of the topsoil was low with a mean of 1.1 % (see Supplement Table 2). An area covered approximately 100 by 60 meters. Per area and time point, 30 samples at a depth of 30 cm were collected using a grid-like sampling along three transects. Each transect was approx. 20 metres apart and with 10 samples collected from each at 10 meter intervals. Soil sampling was as described previously (Kaestli *et al.*, 2009). If grasses were present at the soil sampling points, these were collected in plastic bags and processed within 24 hours. Various abiotic factors were recorded such as soil pH (soil pH field kit, Inoculo, Australia), presence of water logging; distance to closest stream and road drainage, soil water status and soil texture following the "Australian Soil and Land Survey" Field Handbook (McDonald *et al.*, 1998) and a common soil texture flowchart (http://www.h2ou.com/h2twss96.htm) as well as soil color using the Munsell Soil Color Chart (ed. 2009). Using a soil moisture field meter (MPM160 meter, ICT International Pty Ltd, Australia) on a selection of soil samples "moist to wet soil" corresponded to approx. >200 mV or >4% vol soil water content. Soil chemistry

analysis was performed on 18 key soil samples collected in the dry season from sites B, E and P (CSBP Soil and Plant Analysis Laboratory, Bibra Lake, Australia)(see Supplement Table 2).

Sampling started in the dry season, in September 2009 and was repeated three times at 6 month intervals, this covered two consecutive dry seasons and wet seasons. In the wet season, the sampling points closest to the stream at each site were inaccessible due to flooding. In summary, 390 samples were collected from four sites at four time points over 1.5 years.

Inoculation of grasses with *B. pseudomallei*

Seeds of *Pennisetum pedicellatum* were collected from seeding Annual Mission Grass in rural Darwin. Seeds of *Sorghum intrans* and *Oryza rufipogon* were obtained from Greening Australia and of *Brachiaria humidicola cv* Tully from Southedge Seeds (Australia). No *B. pseudomallei* were detected in these seeds by direct *B. pseudomallei* molecular detection (Kaestli *et al.*, 2007). The seeds were germinated on moist sterile cotton or in commercially available potting mix. Once germinated, the seedlings were inoculated with *B. pseudomallei* by soaking only the roots in 2×10e7 CFU *B. pseudomallei*/mL sterile water for one hour after which seedlings were transferred into soil with a soil moisture content of 15 %. The soil represented a soil type common to the Top End, namely sandy clay loam collected from rural Darwin and negative for *B. pseudomallei* by culture and direct molecular detection (Kaestli *et al.*, 2007). The seedlings were grown in closed containers (phytatrays, Sigma-Aldrich Australia) under fluorescent light at 35°C for 12 hours and in darkness for 12 hours at 30°C. Inoculation experiments were conducted with *B. pseudomallei* strain MSHR2817 which was isolated from the rhizosphere of Tully Grass in rural Darwin (ST 144). *B. pseudomallei* strains with ST144 were previously isolated from environmental and clinical sources in the Darwin area.

Comparison of plant growth upon inoculation with *B. pseudomallei*—Seedlings of Tully Grass and annual Mission Grass as well as Wild Rice were divided into three groups; these are negative controls without *B. pseudomallei* inoculation, low (2×10e4 CFU *B. pseudomallei*/mL sterile water) and high (2×10e7 CFU *B. pseudomallei*/mL sterile water) inoculum. For Tully Grass, these groups contained 17, 15 and 15 seedlings (for negative control, low and high inocula), for Mission Grass 24, 19 and 19 and Wild Rice 9, 12 and 10 seedlings. Seedlings were grown in sandy clay loam with a soil moisture content of 15 %. Length of roots and leaves of every plant was recorded before and four weeks after inoculation as well as the appearance of the plant. There was no significant difference between the length of roots or leaves in the different groups at start of experiment. Four weeks after inoculation, 3 plants were combined per group and *Burkholderia* and *B. pseudomallei* load was measured in surface sterilized roots and leaves.

Surface sterilization of leaves and roots—Leaves and roots were detached from the plants, washed in sterile water and surface sterilized by 3 min incubation in 70 % ethanol followed by 1 min in 1 % commercial bleach containing 0.01 % Tween and three washing steps in sterile water (Compant *et al.*, 2008). Water of the initial washing step before sterilization and the last washing step after sterilization was cultured on Ashdown's plates to check for bacterial growth (Ashdown, 1979).

Surface sterilization of leaves was successful with no *B. pseudomallei* colonies detectable in the last washing step while root surface sterilization was successful or resulted in a 10-to 100-fold reduction of *B. pseudomallei* colonies.

Soil and plant DNA extraction

Soil DNA extraction was done as previously described (Kaestli *et al.*, 2009). Briefly, 20 g of soil was incubated with 20 mL of Ashdown's Broth for 39 hours shaking at 37°C, the soil supernatant was centrifuged twice and the pellet processed using the PowerSoil Kit (MoBio Laboratories, USA). Modifications included the addition of 0.8 mg of aurintricarboxylic acid (ATA) and 20 μL of proteinase K (20 mg/mL).

Leaves and roots from field plants were washed with sterile water and 70 % ethanol, cut into fine pieces and processed as above. Leaves and roots from culture plants inoculated with *B. pseudomallei* were surface sterilized as described in previous section, cut into fine pieces and incubated overnight in 10 mL of sterile water, shaking at 37°C. The supernatant was processed as above.

Detection of *B. pseudomallei* DNA by TTS1 real-time PCR

B. pseudomallei DNA was targeted by the well validated *B. pseudomallei* specific TTS1 real-time PCR as described previously (Kaestli *et al.*, 2007; Novak *et al.*, 2006). The limit of detection of this PCR was 15 fg of genomic DNA of *B. pseudomallei* isolate MSHR186 and sensitivity and specificity were found to be 100% based upon screening 224 *B. pseudomallei* and 151 non-*B. pseudomallei* culture isolates. A subset of soil samples was also cultured (Ashdown *et al.*, 1992) and subject to MLST (Godoy *et al.*, 2003).

Semi-Quantification of *B. pseudomallei* load

In order to semi-quantify and compare *B. pseudomallei* load between different leaf or root samples, an internal control plasmid (pT7SsCl) was included into the DNA extraction step to check for differences in DNA extraction efficiency and presence of PCR inhibitors. 2.9 pg of a pT7TS vector with a 1,470 bp insert of a chloride channel of *Sarcoptes scabiei* (kindly provided by Kate Mounsey and Deborah Holt (Mounsey *et al.*, 2007)) were added to the 24 hours enriched plant pellets prior to DNA extraction. In a multiplex PCR targeting TTS1 and pT7SsCl, primers pT7SsCl_forw (final 80 nM; 5′-GTTTTTGACACCATCGCAAGATCG-3′), pT7SsCl_rev (final 100nM; 5′-GTTGTGGTTGATGAGAGATCAGAGA-3′) and the probe pT7SsCl_probe (final 256 nM; 5′-TCGCTCCCAGACGTTTCAGCAGTAACT-3′ labeled with CAL Fluor Red and BHQ-2 (Biosearch Technologies)) were used targeting a 135 bp fragment of the internal control plasmid pT7SsCl. Primer concentrations for pT7SsCl amplification were determined by primer matrix assays and no interference with the TTS1 amplification was found. pT7SsCl standard curves were linear over a dilution series of 6 logs with R^2 of 0.996 and a detection limit of less than 10 plasmid copies. No significant difference was found in recovery rates of pT7SsCl between different grass species nor between roots or aerial parts. TTS1 copy numbers were divided by corresponding pT7SsCl copy numbers to normalize samples for potential differences in extraction efficiency or presence of PCR inhibitors.

Semi-quantification of *Burkholderia* by *rec*A SYBR assay

Bacteria belonging to the genus *Burkholderia* were semi-quantified using a SYBR assay based on a previously published PCR targeting 385 bp of the *rec*A gene (Payne *et al.*, 2005). 4 uL of root- and foliage- extracted DNA were added to final 10 uL PCR mix. 1x TaqMan® Environmental Master Mix 2.0 (Applied Biosystems), 1x SYBR Green 1 solution (Quantace), 400 ng/μL non-acetylated BSA and 900 nM concentrations of primers Burk3 and Burk4 were used. PCR conditions in a Applied Biosystems 7900 system were 50°C 2 min, 95 °C 10 min, followed by 45 cycles of 95 °C 30 sec, 48 °C 1 min, 72 °C 1 min and a final extension step of 72 °C for 10 min. Dissociation curve analysis was from 60°C – 95 °C, increasing 2 °C/sec. The assay was validated using a standard curve with a dilution

series of genomic DNA of *B. pseudomallei* strain MSHR305 over 6 logs and a limit of detection of 475 fg DNA was found and a R^2 of 0.996.

Fluorescent *In Situ* Hybridization (F*IS*H) and Confocal Laser Scanning Microscopy(CSLM)

Leaves and roots were dispatched from plants, fixed overnight in 3% paraformaldehyde at 4°C and stored at −20°C in 50% ethanol as described previously (Amann *et al.*, 1990b). For F*IS*H analysis, a mix of three 16S-rRNA targeted probes (Eurofins MWG Operon, Germany) were used, labeled at the 5′ end with the fluorescent dyes Fluorescein (green), Cy3 (red) or Cy5 (blue): the *B. pseudomallei* specific probe BmBpm-463 (5′-ATCCACTCCGGGTATTAG-3′) [together with the unlabeled competitor Bt-463 specific for *B. thailandensis* (5P′-ATCCACCCCGGTTATTAG-3′)], BET-42a targeting β-Proteobacteria (Manz *et al.*, 1992) with unlabeled competitor GAM-42a targeting γ-Proteobacteria and as negative control for nonspecific probe or dye binding, the probe Non-EUB-338-I which is reverse complementary to the universal bacterial probe EUB338-I (Amann *et al.*, 1990a). Validation of BmBpm-463 in conjunction with Bt-463 was performed *in silico* using the tool probe match implemented in the software package ARB (Ludwig *e t a l.*, 2004) (www.arb-home.de) and the SILVA database SSURef_100_SILVA_02_08_09_opt.arb (Pruesse *et al.*, 2007) (www.arb-silva.de) and by performing F*IS*H with cultured and fixed strains using 10 different *B. pseudomallei* strains of clinical and environmental origin as well as 2 *B. thailandensis*, 1 *B. thailandensis*-like (MSMB43), 1 *B. cepacia*, 1 *B. pyrrocinia*, 2 *Burkholderia* cluster A and B spp., 1 *Cupriavidus* spp. and 1 *Ralstonia* spp. strain. Sensitivity and specificity were 100%. BmBpm-463 is not able to distinguish between *B. pseudomallei* and *B. mallei* 16S rRNA; however, as *B. mallei* did not occur in our target environment, this did not impact on the study.

Hybridizations were carried out on epoxy resin coated glass slides as described previously (Stoffels *et al.*, 2001). Briefly, roots and leaves were cut into fine pieces, dehydrated in ethanol series, air dried and incubated at 46°C in a hybridization solution containing 35% formamide, 30 ng/µl Cy3- and Cy5- and 50 ng/µl Fluorescein-labeled probes and 30 ng/µl of the respective competitors. Washing and mounting of samples was as per (Stoffels *et al.*, 2001).

For microscopy, a Zeiss LSM-510-META confocal laser-scanning microscope (Zeiss, Germany) was used (Stoffels *et al.*, 2001). An argon ion laser supplied a wavelength of 488 nm to excite fluorescein, and two helium neon lasers provided the wavelengths of 543 nm for Cy3 and 633 nm for Cy5.

Statistical Analysis

Statistical analysis was carried out using Stata (Intercooled Stata, version 8.2, USA). For bivariate analysis, Fisher's Exact test and Mann-Whitney U test were used. For multivariable analysis, odds ratios were calculated in stepwise multivariable logistic regression analyses clustered by site. The specification of the models was assessed using a link test and model performance by a Pearson goodness-of-fit test. For spatial autocorrelation analysis, Geary's c statistics were used. All tests were 2-tailed, significant results indicated a P value of <0.01.

Probabilities for exotic grasses and moist soil at field site E were mapped using the geo-statistical interpolation technique "indicator kriging" in ArcGIS9 (ESRI, 2008) with a square grid of 10×10 metres. For the exotic grass data, an anisotropic spherical model was fitted resulting in a semi-variogram with a minor correlation range of 66 metres, a nugget size of 0, a mean prediction error of 0.0034 and average standard error of 0.174. For soil moisture,

an anisotropic exponential model was fitted with a minor correlation range of 59 metres, a nugget size of 0.031, a mean prediction error of –0.0006 and average standard error of 0.279.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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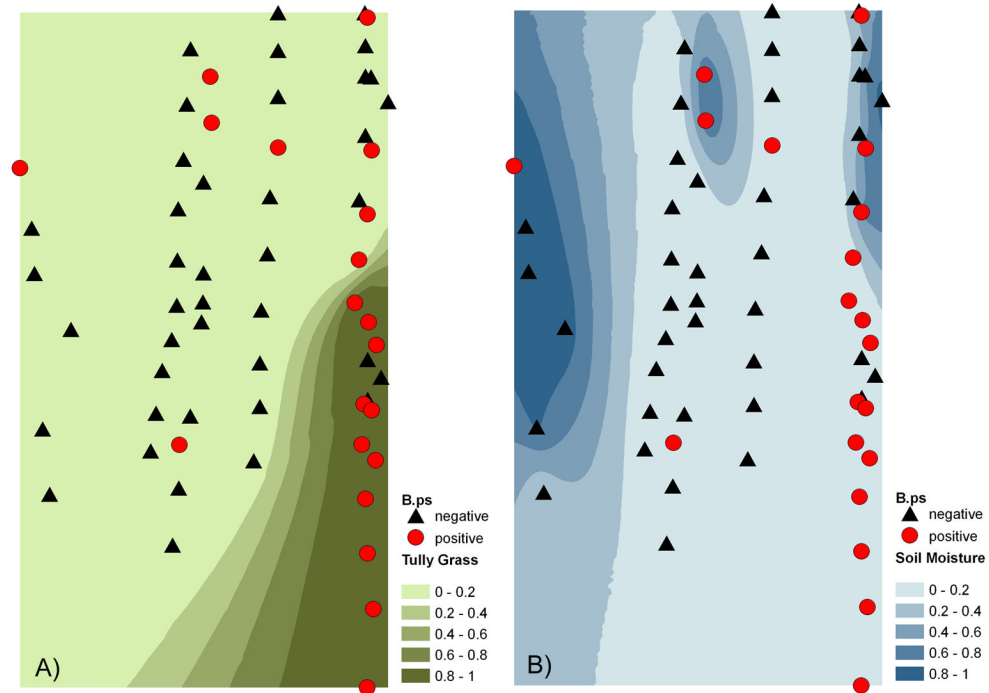
Figure 1.
At field site E which was rich in exotic Tully Grass, soil samples positive for *B. pseudomallei* are shown together with clusters of Tully Grass (A) or moist soil (B). The circles indicate the results of soil screening for presence of *B. pseudomallei* over two consecutive dry seasons. A) The cluster of Tully Grass along the southeast edge is shown by a map based on Indicator Kriging which predicts the probability that the threshold value 0 for no presence of Tully Grass is exceeded. B) Probability map for moist to wet soil (approx. >200 mV or >4% vol soil water content).
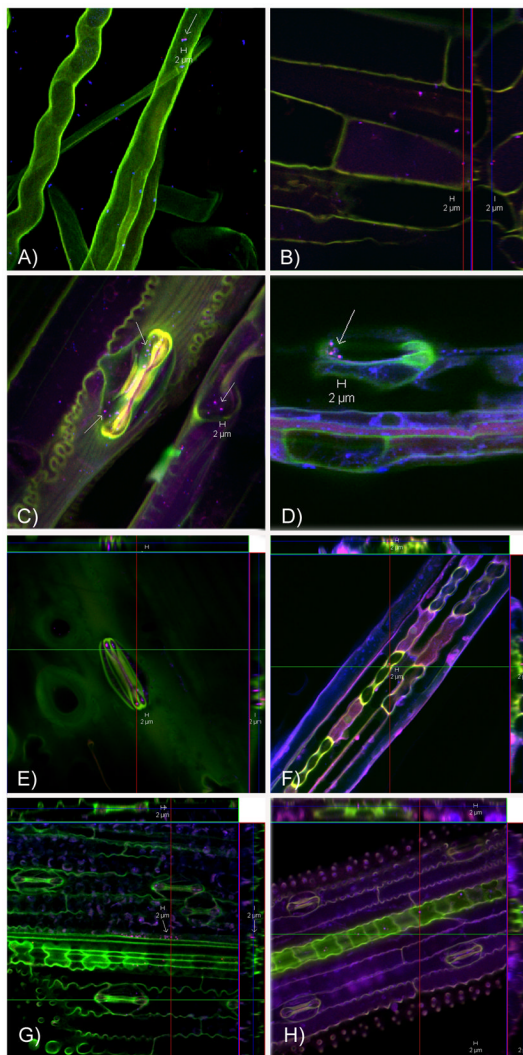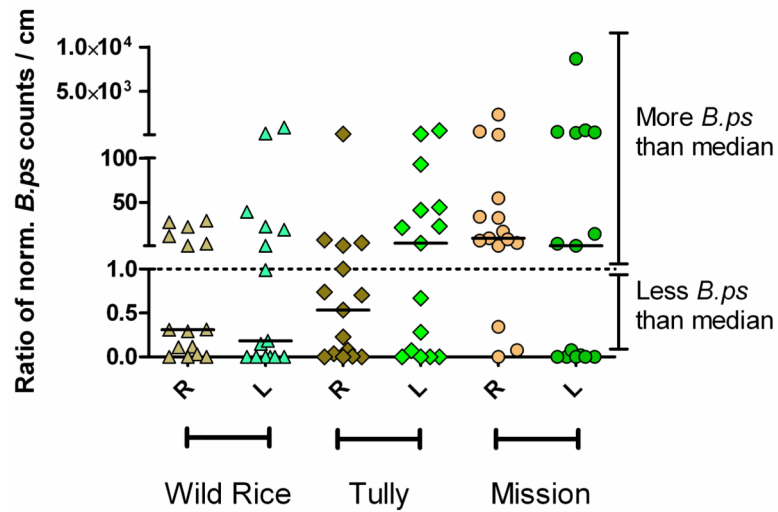
Figure 2.
F*IS*H and confocal laser-scanning microscopy on roots and leaves using a validated set of
fluorescently labeled oligonucleotides targeting the 16S rRNA of *B. pseudomallei* (red); β-
Proteobacteria (blue) as positive control and the probe mix non-EUB-338-I, II, III (green) as
negative control for nonspecific probe or dye binding. The composite pictures are shown
with *B. pseudomallei* cells in magenta (combination of red and blue). No unspecific green
signals were evident for *B. pseudomallei* signals (see Supplement Figure 3). The orthogonal
views (B, E, F, G, H) depict the intra- or extracellular location of *B. pseudomallei* by
showing an internal of three dimensional z-stacks. A) Two *B. pseudomallei* are seen within
a root hair (inside location confirmed by orthogonal view – see Supplement Figure 3) and
further β-Proteobacteria among root hairs of Tully Grass whose rhizosphere was inoculated
with *B. pseudomallei.* B) *B. pseudomallei* in the rhizosphere and inside root cells of Mission
Grass from a highly positive field site. C) Various *B. pseudomallei* and other β-
Proteobacteria are shown close to and within stomatal guard cells of a Mission Grass leaf of
a highly positive field site. D) Three *B. pseudomallei* outside of a stomatal opening of a leaf
of an introduced pasture grass (*Digitaria milanjiana* cultivar Jarra) whose rhizosphere was
inoculated with *B. pseudomallei.* E) *B. pseudomallei* in stomatal guard cells and on the
surface of a stomata of a Paspalum leaf collected at field site B. F)–H) *B. pseudomallei* are

seen along vascular bundles of leaves of grasses whose rhizosphere was inoculated with *B. pseudomallei.* They are at the surface of the lower side of the leaf in a gully-like structure created by the vascular bundles F) Leaf of *Sorghum intrans.* G)–H) Leaves of Wild Rice.

Figure 3.
Semi-quantitative analysis of *B. pseudomallei* load from day three after inoculation in 90 root or leaf samples with 15 samples per group. Samples were of the time series and plant growth comparison experiments. Load comparison was by comparison of ratios with the denominator being the median growth in all root or leaf samples, respectively. *B. pseudomallei* specific TTS1 counts were normalized with an internal plasmid control adjusting for differences in DNA extraction and PCR efficiency. R=roots; L=leaves.

Table 1

Overview of *B. pseudomallei* prevalence in soil and grasses at four field sites

	Sample No. (Soil; Grasses)	Soil around roots % (*B.ps* positive/total samples)				Grass (roots+aerial) % (*B.ps* positive/total samples)			
		Dry Season		Wet Season		Dry Season		Wet Season	
		Nativ	Exot	Nativ	Exot	Nativ	Exot	Nativ	Exot
Total	(380; 217)	20 % **	71 %	31 % **	63 %	10 % **	55 %	12 % **	39 %
Site B Pasp+MG	(106; 53)	44 % **	96 %	59 % **	86 %	13% **	75%	14 % **	67 %
Site E Tully	(103; 61)	19 % **	79 %	31 % **	85 %	8 % **	54 %	8 % *	55 %
Site P Tully	(96; 73)	14 %	40 %	9 %	32 %	5 %	33 %	11 %	15 %
Site S No Exotics	(75; 30)	6 %	na	23 %	na	19 %	na	7 %	na

**
P<0.01 and

*
P<0.05 for difference of prevalence between native and exotic grasses.

B.ps = *B. pseudomallei*; MG=Mission Grass; Pasp=Paspalum; Nativ=native grasses; Exot=exotic grasses

Table 2

(A) Unadjusted and (B) Multivariable logistic regression analysis (odds ratio OR (95% CI) P value) of environmental factors contributing at 5 % significance level to the presence of *B. pseudomallei* in soil at the four field sites in the dry and wet season. Italics in unadjusted models indicate non-significant odds ratios. The following environmental factors were included in the initial model: presence of introduced grass, presence of native spear grass such as *Sorghum* spp., soil pH, soil water status, soil colour, sampling round. Soil texture was consistent both within and between sites and was not included in the analysis (see text and Supplement Table 1). Road drainage was included as a confounder covariate. Controlling for road drainage, the Mantel-Haenszel estimate of the odds ratio of presence of *B. pseudomallei* associated with exotic grasses in the dry season was 4.7 (1.8–12.3) P<0.001. The regression analysis was clustered for sites and the final multivariable models with significant predictor variables were specified correctly as tested by a linktest and the fit of models was declared good (Pearson goodness-of-fit, P>0.36).

	Dry Season(n=213)		Wet Season(n=166)	
	Unadjusted Model	**Multivariable Model**	**Unadjusted Model**	**Multivariable Model**
Exotic Grass	**10.2** (2.8–37.5) P=0.001	**11.7** (3.0–44.7) P<0.001	**3.5** (1.5–8.0) P=0.004	**1.8** (1.1–2.8) P=0.013
Moist to Wet Soil	**5.8** (1.2–28.1) P=0.028	**7.0** (2.2–22.6) P=0.001	*1.2 (0.3–4.3) P=0.779*	
Road Drainage	**8.3** (3.8–18.1) P<0.001		**4.7** (1.6–13.8) P=0.005	**3.3** (1.2–8.8) P=0.019
Soil pH	*1.3 (0.9–2.0) P=0.128*	**1.5** (1.2–1.9) P=0.002	*1.0 (0.7–1.4) P=0.838*	
Soil Color Reddish Grey	*0.5 (0.1–3.6) P=0.531*		*1.8 (0.7–4.6) P=0.242*	**2.6** (1.1–5.8) P=0.025
Soil Color Reddish Yellow	*2.3 (0.6–8.7) P=0.237*		**2.6** (1.3–5.0) P=0.005	**1.3** (1.1–1.4) P<0.001