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1 **Investigating some mechanisms underlying stress metal adaptations of two *Burkholderia sensu lato***
2 **species isolated from New Caledonian ultramafic soils**

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18

19 **Abstract**

20 In New Caledonia, one third of the main island is covered by ultramafic soils characterized by high levels of metals
21 and low levels of plant nutrients. In these soils, metal tolerant bacteria may play a role in plant adaptation to the
22 edaphic constraints. Recently, two new bacteria species belonging to the genus *Burkholderia* sensu lato were isolated
23 from rhizospheric roots of pioneer plant growing in New Caledonian ultramafic soils and were able to tolerate high
24 metal concentrations. The purpose of this study was to determine the major mechanisms by which these two bacteria
25 were able to tolerate metals and if they were able to reduce metal absorption by plants from which they were
26 isolated. Bioaccumulation, adsorption on cell surface, ability to form biofilm and production of exopolysaccharides
27 were assessed. A focus was carried out on Ni, generally the most toxic metal in New Caledonian ultramafic soils. All
28 experiments were performed in comparison with a reference strain isolated from a non-ultramafic habitat,
29 *Paraburkholderia caribensis* MWAP64^T. The two ultramafic bacterial species showed a high tolerance to Ni with a
30 Ni-IC₅₀ of 5 mM for *Caballeronia novacaledonica* STM10272^T and 30 mM for *Paraburkholderia ultramafica*
31 STM10279^T, values significantly higher than those of *P. caribensis* MWAP64^T (2 mM). The major mechanism
32 involved in the bacterial Ni tolerance was related to biofilm formation for *C. novacaledonica* STM10272^T and to
33 acidic exopolysaccharide production for *P. ultramafica* STM10279^T. Both species alleviated metal contents in roots
34 of *Tetradlea comosa*, an endemic pioneer herbaceous species growing on New Caledonian ultramafic soils.

35 **Key words:** Metal tolerance, Biofilm; Exopolysaccharides; *Paraburkholderia*; *Caballeronia*; Plant metal alleviation

36

37

38 1 Introduction

39 Ultramafic soils (also known as serpentinitic soils) are resulting from weathering and pedogenesis of ultramafic
40 bedrocks [1]. These soils are characterized by (i) the dominance of iron oxides with high levels of potentially toxic
41 metals including Ni, Co, Cr and Mn, (ii) low levels of major plant nutrients such as N, P, and K, and (iii) a strong
42 unbalanced calcium/magnesium (Ca/Mg) ratio (<1) [1]. This *a priori* disadvantageous substrate constitutes a specific
43 habitat, which frequently hosts unique plants communities and “clusters” of biodiversity consisting of restricted and
44 endemic taxa [2]. In these extreme edaphic conditions, plants have developed several adaptive mechanisms such as
45 (i) slower growth (ii) ability to limit Mg absorption, and (iii) strategies to tolerate high metal concentrations [2].
46 Among these strategies, the association with specific bacteria play a key role in plant adaptation to harsh edaphic
47 conditions [3,4].

48 The study of microbial communities from ultramafic soils led to the identification of new metal tolerant strains
49 and species [5,6]. Metal tolerant-bacteria show a wide panel of responses towards metals such as biosorption,
50 bioaccumulation, precipitations, chelation, biofilm production, exopolysaccharides (EPS) production or metal
51 transformation, thereby reducing metal toxicity towards themselves [7]. Moreover, metal tolerant bacteria have been
52 shown to reduce the bioavailability of metals and thus to alleviate their toxicity to plants exposed to metal stress [8].

53 In New Caledonia, a tropical archipelago in the South Pacific Ocean, one third of the main island is covered by
54 ultramafic soils. As a result, about 85% of the vascular flora growing on these soils are endemic [9]. However,
55 ultramafic settings are exploited for economically valuable ores such as Ni, Co and Cr, disturbing ultramafic
56 landscapes and classifying New Caledonia among the biodiversity hotspots [10]. New Caledonian ultramafic soils
57 are characterized by high richness and phylogenetic bacterial diversity compared with other environment
58 [11], however the interest for bacteria of these soils is recent [12]. In 2016, two new *Burkholderia* sensu lato (s.l.)
59 species (*Burkholderia novacaledonica* and *Burkholderia ultramafica*), isolated by Gonin et al. (2013) [13] from roots
60 of pioneer plants belonging to the Cyperaceae family growing on ultramafic soils in New Caledonia, have been
61 characterized [6]. Recently, Dobritsa and Samadpour (2019) [14] have proposed the transfer of *B. novacaledonica*
62 STM10272^T and *B. ultramafica* STM10279^T to the genus *Caballeronia* and *Paraburkholderia* respectively.

63 These two species showed tolerance to high levels of the metals found in New Caledonian ultramafic soils [13]
64 suggesting the existence of mechanisms involved in bacterial adaptation to the ultramafic constraint. In this study,
65 we investigated the mechanisms occurred in *Paraburkholderia ultramafica* STM10279^T and *Caballeronia*
66 *novacaledonica* STM10272^T in comparison with *Paraburkholderia caribensis* MWAP64^T isolated from a non-
67 ultramafic soil [15]. We also investigated the implication of *P. ultramafica* STM10279^T and *C. novacaledonica*
68 STM10272^T on the adaptation to ultramafic constraint of *Tetraria comosa*, an endemic pioneer Cyperaceae used in
69 restoration programs [16].

70 2 Material and Methods

71 2.1 Bacterial species

72 For ultramafic species, plant sampling and bacterial strains isolation were performed by Gonin et al. (2013) [13].
73 Full description of *C. novacaledonica* STM10272^T and *P. ultramafica* STM10279^T including morphological,
74 biochemical and molecular characterization was carried out by Guentas et al. (2016) [6] and are reported in
75 Supplementary Table 1. According to Gonin et al. (2013) [13], the two species isolated from ultramafic soils, tolerate
76 edaphic constraints such as low pH, unbalanced Ca/Mg ratio and high metals concentrations (Supplementary Table
77 2). Concerning *P. caribensis* MWAP64^T, bacterial strain was isolated from Vertisol microaggregates in Martinique
78 by Achouak et al. (1999) [15].

79 2.2 Test for metal tolerance (50% inhibition concentration; IC_{50})

80 The metal tolerance was performed in aerobic conditions on microplates (sterile transparent 96 wells PS flat
81 bottom, Greiner) in Lysogenic Broth Lennox medium (LB, tryptone: 10 g L⁻¹, yeast extract 5 g L⁻¹, NaCl: 5g L⁻¹;
82 CONDA, Spain) supplemented with different metals concentrations (3 replicates by plate) as suggested by Chien et
83 al. (2013) [17]. For these experiments, 1 M metal stock solutions were prepared from solid salts (Ni as NiSO₄·6H₂O,
84 Co as CoCl₂·6H₂O, Cr as K₂Cr₂O₇, and Mn as MnSO₄·H₂O). To avoid metal precipitation in the assays, like
85 suggested by Hartley et al. (1997) [18], these stock solutions were sterilized separately. Each well was filled with 20
86 μL of an overnight bacterial culture adjusted to an OD₆₀₀ of 1, (which is approximately equal to 10⁸ CFU mL⁻¹) and
87 180 μL of broth tested. The plate was incubated at 28°C under 125 r min⁻¹ orbital shaking in a microplate incubator

88 reader (EPOCH 2 microplate reader; BioTek Instruments, Winooski, Vermont, USA). Cell growth was monitored by
89 measuring the OD₆₀₀ every hour for 48 h. The experimentations were done at least twice independently. According to
90 the preliminary result of Gonin et al. (2013) [13], metals concentration tested ranged from 0.5 to 100 mM for Ni and
91 Mn and from 0.01 to 50 mM for Co and Cr. The strain growth rates (μ) were determined for each concentration. A
92 sigmoid dose–response curve was obtained by plotting growth rate against metal concentrations, and IC₅₀ values
93 were determined using GraphPad Prism software (version 6, GraphPad Software, La Jolla California USA).

94 2.3 *Ni biosorption and bioaccumulation*

95 The metal adsorbed onto the surface of the bacterial cell and the metal accumulated inside the cell were
96 determined according to Giovanella et al. (2017) [19], with modifications as follows. A primary culture was done by
97 inoculating 10 mL of LB Lennox medium with one colony of the chosen bacteria and then incubating for 24 h at
98 28°C and 125 r min⁻¹. A culture (25 mL) inoculated at an OD₆₀₀ of 0.1 by a primary culture was incubated for 24 h at
99 28°C under rotary shaking at 125 r min⁻¹; then the culture was artificially contaminated by aseptically adding Ni
100 such that Ni reached a final concentration of 1 mM, 5 mM and 50 mM (based on Ni-IC₅₀ values). After 24 h, 1 mL
101 samples were removed from the culture and centrifuged at 13,000 r min⁻¹ for 10 min (Sigma, 2-16K). The pellet was
102 washed thrice in sterile deionized water to remove free metal ions and finally suspended in 1 mL of sterile deionized
103 water. The OD₆₀₀ was read and the number of colony-forming units (CFU) per milliliter of the inoculum was carried
104 out. To determine Ni accumulation in the cell walls and intracellular spaces of these bacteria, another aliquot (1 mL)
105 was centrifuged at 13,000 r min⁻¹ for 10 min; the pellet was washed with ethylenediaminetetraacetic acid (EDTA, 10
106 mM, pH: 6.5) for Ni desorption of from the cell surface, centrifuged, and re-suspended in 1 mL of sterile deionized
107 water. Finally, the total amount of Ni accumulated intracellularly (bioaccumulation) and sorbed onto the cells
108 (biosorption) was determined by digesting the 1 mL aliquots in a nitric acid (69%, 1 mL) and hydrogen peroxide
109 (30%, 0.5 mL) solution for 1 night at room temperature. The digested samples were then adjusted to 10 mL with
110 deionized water, and the Ni content in the final solution was quantified by Inductively Coupled Plasma Optical
111 Emission Spectrometry (ICP OES, Varian, Varian 730–ES, Palo Alto, USA) at Laboratoire des Moyens Analytiques
112 (LAMA-US IMAGO-IRD, New Caledonia). The amount of Ni accumulated intracellularly (bioaccumulation) was
113 determined by subtracting the Ni content adsorbed onto the cell walls (biosorption) from the total Ni amount.

114

115 2.4 *Biofilm formation assay in the presence of Ni*

116 The biofilm formation assay was performed on a microtiter plate [20]. Aliquots of 90 μL of LB culture medium
117 supplemented with different concentrations of Ni [(as $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$)] to reach 0.5, 1, 2.5, 5, 8, and 10 mM Ni
118 concentration (based on Ni- IC_{50} values) were loaded into 96-well microtiter plates (sterile transparent 96 wells PS
119 flat bottom, Greiner). Wells were inoculated with 10 μL of a 48 h primary culture adjusted to an OD_{600} of 1. For
120 these experiments, a non-inoculated LB medium was used as the negative control, whereas an inoculated LB culture
121 medium without Ni was used as the positive control. After, 24 h, 48 h, and 72 h of incubation at 28°C without
122 agitation, the medium and the non-adhering bacteria biofilm were eliminated by simple inversion and each well was
123 washed three times with 100 μL of sterile deionized water. The microplates were then dried at 50°C for 30 min. The
124 cells from the adhering biofilm were dyed with 100 μL of 0.02% crystal violet (CV) solution (30 min at room
125 temperature). The excess of dye was removed by simple inversion and the biofilm was washed three times with 100
126 μL of sterile deionized water at each wash. The microplates were dried at room temperature for 10 min before
127 resuspending CV by adding 200 μL of 95% ethanol solution. After 10 min orbital shaking at 125 r min^{-1} , the content
128 of each well was transferred into a new microplate and the OD_{590} was read with a microplate reader (EPOCH 2
129 microplate reader; BioTek Instruments, Winooski, Vermont, USA). The results obtained were then transformed into
130 a quantification of biofilm formation by calculating the average absorbance of six replicates for each species and for
131 each concentration and dividing by the average absorbance of the blank (without bacteria). The results expressed as
132 this ratio can be considered as non-dependent from the non-specific coloring of the surface of the wells of the
133 microplates by the CV. Following this procedure, biofilm formation was considered to have occurred when the ratio
134 is greater than 2 [21].

135 2.5 *Quantification of biofilm formation*

136 Since the concentration of bioavailable Ni frequently reaches 1 mM in ultramafic soils, especially in the
137 rhizosphere from New Caledonia [22], quantification of biofilm production without Ni and at 1 mM of Ni was
138 carried out. The biofilm formation was visualized by confocal laser scanning microscopy (CLSM) after 24 h. For
139 these experiments, a 24 h primary culture was first adjusted at a 0.1 OD_{600} in LB medium and used to inoculate a 24-

140 wells in glass bottom opaque black screening plate (Sensoplate, Greiner Bio-One) (1 mL per well). The microtiter
141 dish was then incubated for 24 h and the biofilms were stained for 10 min with 1% of Syto 61 red fluorescent nucleic
142 acid stain (5 mM, Molecular Probes, USA). CLSM observations were performed with a TCS-SP2 microscope
143 (Leica Microsystems, Heidelberg, Germany), using a 63x immersion objective. The excitation and Emission
144 wavelengths for Syto 61 Red were set at 633 nm and 645 nm, respectively. The images collected by confocal stacks
145 were processed using Leica Confocal Software and the biovolume, thickness and substratum coverage of biofilms
146 were quantified with COMSTAT [23].

147 2.6 Production and gross chemical composition of EPS

148 Regarding its ability to produce an exopolysaccharide [15], *P. caribensis* MWAP64^T was used as a positive
149 control in order to approve the experimental design. EPS production was quantified after 96 h of culture at 28°C
150 under 125 r min⁻¹ orbital shaking in minimum broth medium (MB; KH₂PO₄ · 3H₂O, 0.66 g L⁻¹; NaCl, 50 mg L⁻¹;
151 MgSO₄ · 7 H₂O, 10 mg L⁻¹; FeCl₃, 4 mg L⁻¹; CaCl₂, 40 mg L⁻¹) supplemented with glucose (30 g L⁻¹). In order to
152 produce biomass, 8 L of LB were inoculated using a 48 h grown culture (800 mL; 28°C, 125 r min⁻¹ orbital shaking).
153 After 72 h of incubation (28°C, 125 r min⁻¹ orbital shaking), the biomass was recovered by centrifugation at 9,000 r
154 min⁻¹ (13,700 g) for 25 min (Sorvall RC5C Centrifuge, DuPont) and washed once with sterile deionized water. Cells
155 were transferred at 28°C to 10 L reactors containing 8 L of MB medium supplemented with glucose. The bacteria
156 were removed from the medium by centrifugation at 9,000 r min⁻¹ (18,894 g) for 25 min. The supernatants were
157 collected and the EPS were purified by ultrafiltration on a 100,000 molecular weight cut-off membrane from
158 Sartorius and immediately freeze-dried. Protein content was determined by the Lowry method [24], modified by
159 Smith et al. (1985) [25] and Brown et al. (1989) [26]. Sugar contents, was quantified by the method described by
160 DuBois et al. (1956) [27] and completed by Chaplin (1986) [28], and uronic acid contents by the Blumenkrantz and
161 Asboe-Hansen method [29], modified by Filisetti-Cozzi and Carpita (1991) [30]. Infrared spectroscopy was used to
162 complete the biochemical characterization of EPS. The infrared spectrum of the freeze-dried EPS was recorded using
163 a Fourier Transform Infrared (FT-IR) spectrometer Frontier TM (Perkin Elmer) at the spectroscopy lab of the IRD
164 center in Noumea (New Caledonia). This spectrum corresponds to an average of four successive spectra that were

165 collected in Attenuated Total Reflectance mode (diamond crystal) over the 500 cm⁻¹ – 3500 cm⁻¹ range with a 4 cm⁻¹
166 resolution.

167

168

169 2.7 Effect of microbial inoculation on *Tetradlea comosa* plants under ultramafic conditions

170 The inocula for pot trial were prepared as follows: A pre-inoculum was performed as described previously in the
171 section 2.3. After 2 days, the aerobic cultures were used to inoculate 100 mL of LB media at an OD_{600 nm} of 0.1.
172 After 3 days at 28°C under orbital agitation (125 r min⁻¹), the cells were pelleted by centrifugation (SIGMA®, 2K15,
173 5,000 g, 20 min), washed twice with sterile deionized water and, then re-suspended in sterile deionized water at a
174 bacterial concentration between 10⁷ and 10⁸ CFU mL⁻¹ (corresponding to an OD₆₀₀ of 0.5), as suggested by Larcher
175 et al. (2003) [31]. The purity was checked and the concentration of the inoculum was carried out after a bacterial
176 count on LB agar plate.

177 SIRAS Pacifique (Noumea, New Caledonia) provided seeds of *Tetradlea comosa*. Germination occurred on a
178 compost amended ultramafic soil composed of (i) 2 mm sieved colluvial lateritic soil (ferralsol) sampled in Plum
179 area in New Caledonia (22°16'59''S, 166°39'12''E) and (ii) commercial compost (4:1 v:v). Characteristics of the
180 ferralsol are: coarse sand, 39.4%; fine sand, 22.1%; silt-clay, 37.2%; pH H₂O, 5.9; pH KCl, 5.6; total C, 42.1 g kg⁻¹;
181 total N, 2.2 g kg⁻¹; total P, 147 mg kg⁻¹; available P (Mehlich), 3 mg kg⁻¹; total Ca, 1.06 g kg⁻¹; total Mg, 5.08 g kg⁻¹
182 and Ca/Mg, 0.207. The total metals contents of the ferralsol measured after an alkaline fusion are as follows: Co,
183 0.87 g kg⁻¹; Cr, 22 g kg⁻¹; Fe, 348 g kg⁻¹; Mn, 10 g kg⁻¹; Ni, 5.6 g kg⁻¹. The available metals extracted by
184 dimethylene triaminopentaacetic acid (DTPA) are as follows: Co_{DTPA}, 68 mg kg⁻¹; Cr_{DTPA}, 0.17 mg kg⁻¹; Fe_{DTPA}, 71
185 mg kg⁻¹; Mn_{DTPA}, 1.1 g kg⁻¹; Ni_{DTPA}, 130 mg kg⁻¹. The composition of commercial compost is as follows: N, 1.7
186 mg g⁻¹; P_{total}, 150 mg kg⁻¹; P_{Olsen}, 7 mg kg⁻¹; K, 139 mg kg⁻¹ (Terreau universel, Agrofino, France). The compost
187 amended-ultramafic soil was autoclaved 3 times at 120°C for 1 h, with an interval of 24 h before being used in the
188 greenhouse experiment. Although, *T. comosa* was able to develop on ultramafic soil in natural condition, the addition
189 of this commercial compost to the ultramafic soil was necessary because the growth of the plantlets of *T. comosa*

190 could be inhibited under greenhouse condition in a pure sterile lateritic topsoil [32]. After two months, roots
191 seedlings were washed with sterile water to remove any contaminants and were transplanted into one-liter plastic
192 containers filled with the compost amended-ultramafic soil described above. A 5 mL bacterial inoculum was applied
193 on roots of each plantlet during transplantation in 1 L pots. The same volume of sterilized water was applied to a
194 control set of plants. A set of 15 plants was inoculated per treatment. The plants were grown under greenhouse
195 conditions as reported by Lagrange et al. (2011) [33]. After 15 months of growth, the plants were harvested. Shoot
196 and root biomasses were determined by measuring the dry mass tissue (3 days at 60°C). The 15 samples of plant
197 tissues were randomly pooled by five and ground to powder. The contents of P, K, Ca, Mg, Co, Cr, Mn, and Ni in
198 tissues were quantified by ICP OES with a Varian 730-ES spectrometer after digestion in a mixture (4:1, v/v) of
199 HNO₃ (69%) and H₂SO₄ (37%) at the Laboratoire des Moyens Analytiques (LAMA-US IMAGO-IRD, New
200 Caledonia).

201 2.8 Statistical analysis

202 All statistical analysis were performed with the R software version 3.3.1 [34]. All data were analyzed with
203 parametric tests (one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference
204 (HSD) test; $P < 0.05$) or with non-parametric tests (Kruskal-Wallis test; followed by the Fisher's least significant
205 difference (LSD) test; $P < 0.05$) when data did not follow the assumptions of a parametric test.

206 3 Results

207 3.1 Test for metal tolerance

208 The metals IC₅₀ determined for the three species are reported in Table 1. *P. ultramafica* STM10279^T and *C.*
209 *novacaledonica* STM10272^T showed a significant higher tolerance to Co, Mn and Ni compared to *P. caribensis*
210 MWAP64^T. For instance, *P. ultramafica* STM10279^T Ni-IC₅₀ value was 16 times significantly higher and *C.*
211 *novacaledonica* STM10272^T Ni-IC₅₀ value was 2.5 times significantly higher than Ni-IC₅₀ value of *P. caribensis*
212 MWAP64^T. The two ultramafic species showed significantly different metal tolerance profiles for Cr and Ni. Indeed,
213 the Ni IC₅₀ of *P. ultramafica* STM10279^T was 6.4 times higher compared to Ni-IC₅₀ value of *C. novacaledonica*
214 STM10272^T. Conversely, the Cr IC₅₀ of *P. ultramafica* STM10279^T was significantly lower than Cr-IC₅₀ value of *C.*

215 *novacaledonica* STM10272^T. No significant differences between the two ultramafic species were observed for the
216 tolerance to Co and Mn.

217 3.2 *Ni biosorption and bioaccumulation*

218 Biosorption of Ni onto the cell surface and intracellular compartment were evaluated. Results expressed in ng
219 (10^7 CFU)⁻¹ are reported in Fig. 1. The fraction of Ni inside the cells and onto cell wall surface were significantly
220 lower for *P. ultramafica* STM10279^T and *C. novacaledonica* STM10272^T than for *P. caribensis* MWAP64^T.
221 Moreover, significant differences are observed between *P. ultramafica* STM10279^T and *C. novacaledonica*
222 STM10272^T. Major differences appeared at 5 mM of initial Ni concentration, the mean intracellular Ni content in *P.*
223 *ultramafica* STM10279^T (0.76 ± 0.02 ng (10^7 CFU)⁻¹) was 430 times lower than the value of *C. novacaledonica*
224 STM10272^T and was 11,000 times lower compared to the value of *P. caribensis* MWAP64^T. Ni content on cell
225 surface of *P. ultramafica* STM10279^T (3.45 ± 0.34 ng (10^7 CFU)⁻¹) was 86 times lower than the value of *C.*
226 *novacaledonica* STM10272^T and was significantly 675 times lower compared to the value of *P. caribensis*
227 MWAP64^T.

228 3.3 *Biofilm formation assay in the presence of Ni*

229 Biofilm formation by the three species studied in the presence of different Ni concentrations and their variation
230 with time are presented in Fig. 2. At 24 h increase of Ni concentration decreased biofilm formation for all the strains
231 then, biofilm formation increased with time particularly for *C. novacaledonica* STM10272^T. At 48 h and 72h
232 biofilm formation occurred until 2.5 mM of Ni initial concentration for *P. ultramafica* STM10279^T and until 1 mM
233 of Ni initial concentration for *C. novacaledonica* STM10272^T and *P. caribensis* MWAP64^T. The amount of biofilm
234 formed by *C. novacaledonica* STM10272^T was significantly higher than those formed by *P. ultramafica* STM10279^T
235 and *P. caribensis* MWAP64^T. The differences found ranged from 1.8 times, 2.6 times and 3 times higher at
236 respectively 0, 0.5 and 1 mM initial Ni concentration at 48 h and 72 h, in comparison to the control. At 48 h and 72 h
237 initial Ni addition did not influence biofilm formation by *C. novacaledonica* STM10272^T until 2.5 mM. At the
238 opposite, biofilm formation by *P. ultramafica* STM10279^T and *P. caribensis* MWAP64^T decreased with the increase
239 of Ni concentration until 2.5 mM and 1 mM of Ni for *P. ultramafica* STM10279^T and *P. caribensis* MWAP64^T
240 respectively.

241 **3.4** *Quantification of biofilm production*

242 The biovolume, thickness and substratum coverage of the biofilms produced by the three species studied are
243 presented in Fig. 3. The biovolume, the thickness average and the substratum coverage of the biofilm produced by *P.*
244 *ultramafica* STM10279^T and *C. novacaledonica* STM10272^T were significantly higher compared to the biofilm of *P.*
245 *caribensis* MWAP64^T. Moreover, the addition of 1 mM of Ni to the medium has significantly reduced the substratum
246 coverage by *P. caribensis* MWAP64^T biofilm from 60% to 39%. For *C. novacaledonica* STM10272^T, the addition of
247 Ni has significantly enhanced the thickness average of the biofilm from 21 μm to 26 μm .

248 **3.5** *EPS production and its gross chemical composition*

249 Under the experimental conditions used in this study, *P. caribensis* MWAP64^T was able to produce EPS at a
250 production yield around 1.2 g L⁻¹. Regarding ultramafic species, only *P. ultramafica* STM10279^T was able to
251 produce EPS at a production yield around 140 mg L⁻¹. FT-IR spectroscopy was employed to detect the main
252 functional groups in the EPS produced by *P. ultramafica* STM10279^T. The FT-IR spectrum obtained (Fig. 4)
253 showed a broad and large absorbance band centered at 3276 cm⁻¹ that was assigned to hydroxyl groups or hydrogen
254 bonds. It also showed two peaks at 2925 and 2868 cm⁻¹ that were respectively assigned to CH-stretching of
255 methylene (-CH₂) and methyl (-CH₃) groups. Several absorbance peaks between 1500 cm⁻¹ and 1760 cm⁻¹ that were
256 assigned to the stretching modes of C=O bonds were also visible. The peak at 1719 cm⁻¹ indicated the presence of
257 carboxyl of uronic acid groups, the peak at 1248 cm⁻¹ is assigned to the deformation vibration of C=O bonds and the
258 peak at 1032 cm⁻¹ is assigned to the stretching modes of C-O from intra-molecular C-O-C ring. This latter peak thus
259 strongly suggested that the monosaccharides in the EPS have pyranose rings. In addition, the two weak peaks near
260 841cm⁻¹ and 896 cm⁻¹ could be respectively assigned to the two α - and β -ends of carbon-glucoside bonds. Finally,
261 two additional peaks could be seen at 783 and 701 cm⁻¹ but they could not be unambiguously assigned to specific
262 molecular groups. The colorimetric test carried out (Supplemental Table 3) has shown that the ratio of carbohydrates
263 to proteins (C/P) was almost 15, with values of 61% carbohydrates to 4% of proteins residues and the presence of
264 uronic acid (17%).

265 **3.6** *Effect of microbial inoculation in Tetraria comosa under ultramafic conditions*

266 Most significant data variations were observed for the contents of metal in plant tissue (Table 2). In shoot, no
267 significant differences were measured between the inoculated plants and the control, but in roots, the three strains
268 reduced metal contents even if no significant differences were observed with the inoculation of *C. novacaledonica*
269 STM10272^T for Co and Mn contents compared with control. The values of the 4 elements were about two times
270 lower in presence of *P. ultramafica* STM10279^T and *P. caribensis* MWAP64^T in comparison with control plants.
271 The effect of *C. novacaledonica* STM10272^T on root metal contents was less pronounced but the differences with *P.*
272 *ultramafica* STM10279^T were not significant. The inoculation of *T. comosa* plants with *P. ultramafica* STM10279^T
273 and *P. caribensis* MWAP64^T did not induce significant changes in plant biomass (Supplemental Table 4) and main
274 nutritional element contents in plant organs (Supplemental Table 5). However, the inoculation with *C.*
275 *novacaledonica* STM10272^T significantly decreased the shoot and root biomasses compared to the other treatments
276 (50% lower in average).

277 **4 Discussion**

278 In our experiments, *C. novacaledonica* STM10272^T and *P. ultramafica* STM10279^T showed a higher metal
279 tolerance than *P. caribensis* MWAP64^T a non-ultramafic species isolated from Vertisol in Martinique [15]. These
280 results are in agreement with previous studies, which have shown that ultramafic soils are known to hold bacterial
281 communities more adapted to metal stress [4]. For instance, a set of *Burkholderia* s.l. strains isolated from Ni-rich
282 ultramafic soil, tolerated up to 15 mM Ni, 10 mM Co and 5 mM Cr [35]. In New Caledonia, Stoppel and Schlegel
283 (1995) [36] reported the occurrence of *Burkholderia* s.l. strains that could tolerate up to 30 mM Ni which is
284 comparable to *P. ultramafica* STM10279^T Ni-IC₅₀.. Differences in profiles of metal tolerance were observed between
285 the two ultramafic species and more particularly for Ni suggesting that different mechanisms are involved depending
286 of the species.

287 Intracellular sequestration has already been reported as a mechanism of Ni tolerance for *Burkholderia* s.l. species
288 [37]. However, our data indicated a preferential biosorption of Ni onto the cell surface of the three species studied.
289 This result suggests that the tolerance mechanism used by these three species was to limit the movement of Ni across
290 the cell envelope. *Burkholderia* s.l. species are gram-negative bacteria that exhibit a complex cell-membrane

291 structure composed of numerous phosphorylated biomolecules (i.e. phospholipids, lipoprotein, lipopolysaccharide)
292 located on the external membrane and involved in metal chelation [38]. In fact, the various components (proteins,
293 exopolysaccharides layers, extracellular capsules) of the surface of bacteria cells are known to provide non-specific
294 sorption capacity toward metals [39]. This non-specific binding capacity of the cell wall has been already
295 demonstrated to prevent the entry of nickel into the bacteria cell and thus to hinder its interference with essential
296 cellular components [7].

297 Biofilm formation is known to be involved in bacterial tolerance to Ni [17,40]. In our study, biofilm production
298 occurred until 1 mM for *C. novacaledonica* STM10272^T and *P. caribensis* MWAP64^T and until 2 mM for *P.*
299 *ultramafica* STM10279^T. It is not surprising that biofilm production was reduced at concentrations below the IC₅₀.
300 Indeed, Vega et al. (2014) [41] have shown that Ni inhibited the *Quorum Sensing* and biofilm formation without
301 affecting viability in *Burkholderia multivorans*. Confocal analysis clearly demonstrated the negative effect of nickel
302 on the formation of biofilm in *P. caribensis* MWAP64^T suggesting that another mechanism must be involved in the
303 metal tolerance. Until 1 mM of Ni, biofilm formation appeared to be an important mechanism for *C. novacaledonica*
304 STM10272^T in view of the large quantities of biofilm found and the enhancement of thickness average with the
305 addition of Ni. Since the concentration of bioavailable Ni frequently reaches 1 mM in New Caledonian ultramafic
306 soils, especially in the rhizosphere [22], we can conclude that Ni-concentration in these soils could induce biofilm
307 formation. These results corroborate those obtained by Perrin et al. (2009) [42] who showed *in vitro* biofilm
308 stimulation by nickel for *Escherichia coli*.

309 In order to go further into the mechanisms developed by the three *Burkholderia* s.l. species, we investigated their
310 ability to excrete EPS. Indeed, the high molecular sugar-based polymers that constitute these bacterial extracellular
311 substances have already been described to play a role in *Burkholderia* s.l. adaptation to different stress conditions
312 [43]. Bacterial EPS are known to play a significant role in the complexation of metals and subsequent decrease in
313 their mobility in soils [39]. Under our experimental conditions, *P. ultramafica* STM10279^T and *P. caribensis*
314 MWAP64^T were able to produce EPS. *P. caribensis* MWAP64^T is a well-known EPS-producing bacterium [15].
315 Structural analysis of the polysaccharide excreted by *P. caribensis* strain MWAP71, a strain belonging to the *P.*
316 *caribensis* cluster [15] has shown the presence of 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) [44]. Anionic groups
317 such as carbonyl, carboxyl and hydroxyl groups have been reported to scavenge several metals and to be involved in

318 metal biosorption [39,45]. Colorimetric assays showing that carbohydrates were the main component in the crude
319 EPS produced by *P. ultramafica* STM10279^T and the FT-IR peaks found suggest that the EPS of *P. ultramafica*
320 STM10279^T is a polysaccharide containing pyranose and uronic acid groups [46–48]. All the features on the FT-IR
321 spectrum of the EPS of *P. ultramafica* STM10279^T indicated that (i) carbohydrates were the main component in the
322 crude EPS produced by *P. ultramafica* STM10279^T and (ii) the EPS produced by *P. ultramafica* STM10279^T was a
323 polymer of a repeating unit presenting uronic acid residues. The pyranose and uronic acid groups that are expected to
324 constitute the polysaccharides are considered involved in the metal binding process. Such a behavior has already
325 been shown for *Rhizobium etli*, which was able to chelate Mn in these EPS [49].

326 The two ultramafic species clearly showed different metal tolerance patterns and mechanisms. *C. novacaledonica*
327 STM10272^T was found to develop a biofilm to protect from metals, whereas *P. ultramafica* STM10279^T excreted
328 EPS to sequester metals, although it was able to develop a biofilm. Finally, our results are in agreement with the
329 literature, as biofilm formation has already been reported for *Paraburkholderia* [50] and *Caballeronia* species [51],
330 while EPS production was only observed in the *Paraburkholderia* genus [15,52,53]. However, other mechanisms
331 may occur in metal tolerance of *P. ultramafica* STM10279^T and *C. novacaledonica* STM10272^T. Gonin et al. (2013)
332 [13] have shown that these bacteria possess *cnr* (Co–Ni resistance) and *nre* (Ni resistance) genes involved in metal
333 exclusion and active removal. Such mechanisms may operate in *C. novacaledonica* STM10272^T and in *P.*
334 *ultramafica* STM10279^T.

335 All these mechanisms put in place by bacteria to protect themselves can be also involved in the variations of
336 metal absorption by inoculated plants [8]. Alleviation of metal contents in plants by metal tolerant *Burkholderia* s.l.
337 have been reported in the literature. Madhaiyan et al. (2007) [54] showed a reduction of Ni uptake and translocation
338 in tomato after inoculation with a *Burkholderia* s.l. strain. These authors suggested that this reduction might be
339 attributed to the biosorption of Ni by the bacterial cells, which would have prevented the metal from being
340 transported into the plant. Kuffner et al. (2010) [55] reported that a *Burkholderia* s.l. strain had the capacity of
341 reducing Zn and Cd accumulation in the roots of *Salix caprea* by favoring the immobilization of these metals in the
342 soil. Our results on the effect of *P. ultramafica* STM10279^T and *C. novacaledonica* STM10272^T are in agreement
343 with these studies. However, the alleviation of metals observed in *T. comosa* were more pronounced with *P.*

344 *ultramafica* STM10279^T than *C. novacaledonica* STM10272^T, in possible relation to the differences of their metal
345 profile tolerance.

346 Although it is important to underline that an increase of the plant biomass is not the unique aspect of plant health
347 and adaptation [56], the lack of positive effect of bacterial inoculation on the biomass of *T. comosa* plants suggests
348 that metal alleviation had no influence on *T. comosa* plant growth. Other adaptation mechanisms inherent in the plant
349 may explain this result. Indeed, Gonin et al. (2013) [13] using sorghum, a plant non-native of ultramafic maquis,
350 reported a significant enhancement of plant biomass when inoculated by *P. ultramafica* STM10279^T. It must be
351 outlined that *Tetraria* species are considered as oligotrophic (with low nutrition requirements) and their low growth
352 rate is an adaptive trait to ultramafic constraints [57], which can also explain the absence of positive effect of
353 bacterial inoculation on their biomass production. Concerning the detrimental effect of *C. novacaledonica*
354 STM10272^T on plant biomass, it can be possibly explained by their ability to produce high level of biofilm. Although
355 biofilm plays a major role in the attachment of bacteria to plant roots, known biofilm-forming rhizosphere
356 *Burkholderia* s.l. are reported to inhibit root development [58].

357 **5 Conclusions**

358 The results of the present study clearly demonstrated that the two ultramafic bacterial species used different
359 mechanisms to cope with metal toxicity such as cell wall absorption, biofilm formation and EPS production. These
360 mechanisms can explain the decrease of metal contents in the plant and underlined the importance to consider the
361 plant/rhizobacteria interaction in ultramafic soils in order to understand the mechanisms occurred in plant adaptation
362 to the extreme edaphic constraints. Although no improvement in plant growth could be noticed, the ability of *P.*
363 *ultramafica* STM10279^T to produce an EPS can create a more hospitable environment for other organisms that will
364 promote plant growth. It will then be interesting to experiment the co-inoculation of the studied bacteria with
365 arbuscular mycorrhizal fungi, to combine effects on metal alleviation in plant organs and improvement of plant
366 mineral nutrition.

367 **Disclosure statement**

368 Declaration of interest: none.

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374 **Appendix A. Supplementary data**

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Fig. 1.

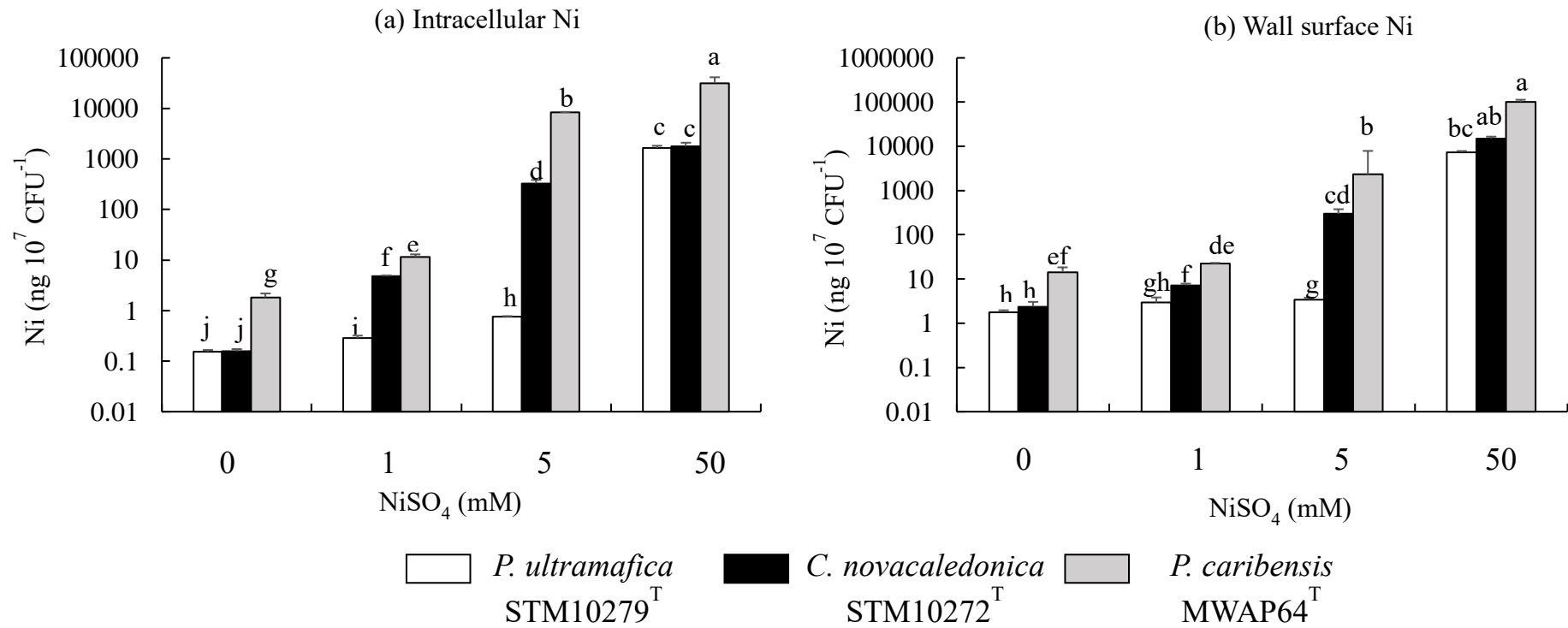
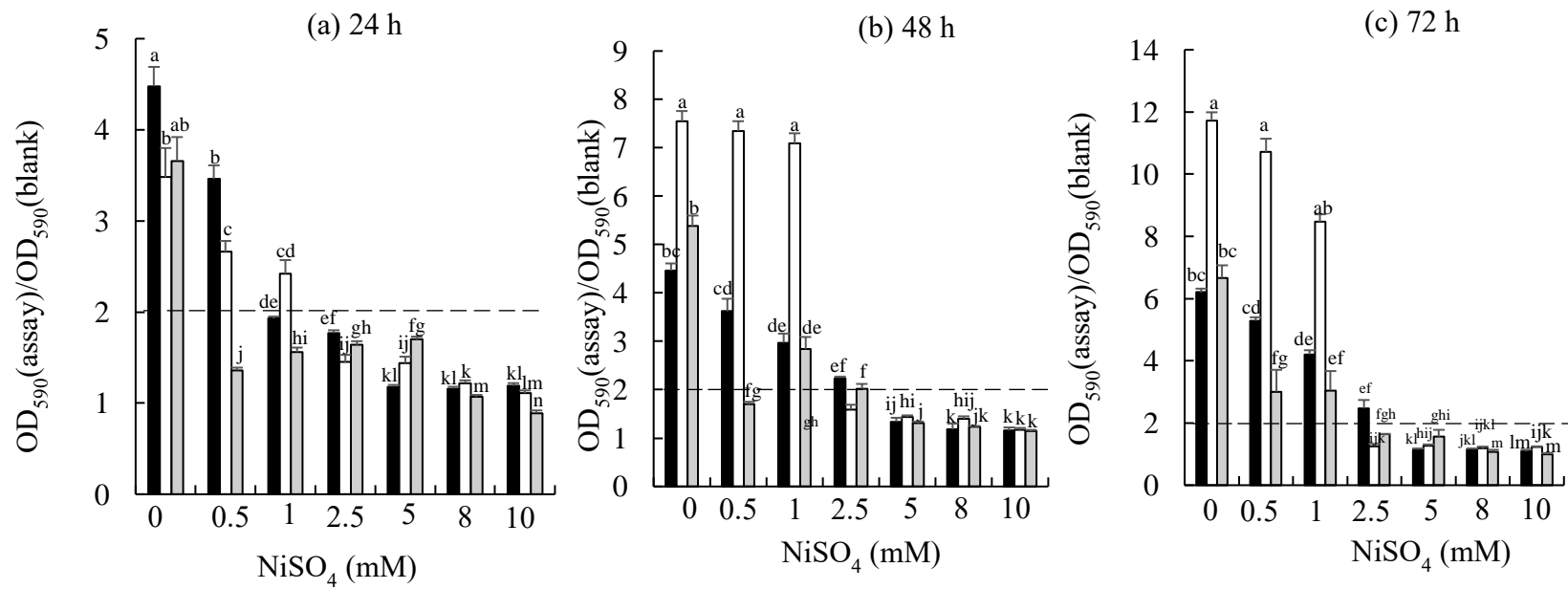


Fig. 2.



P. ultramafica STM10279^T
 C. novacaledonica STM10272^T
 P. caribensis MWAP64^T

Fig. 3.

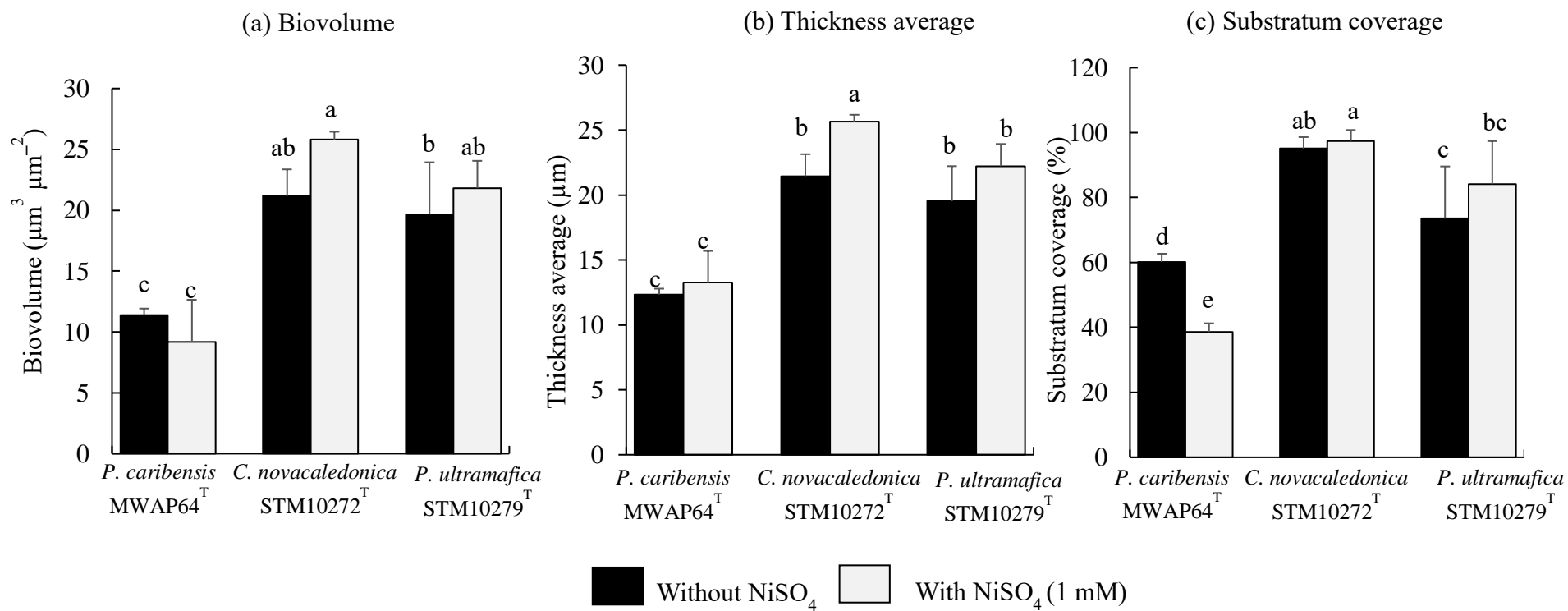


Fig. 4.

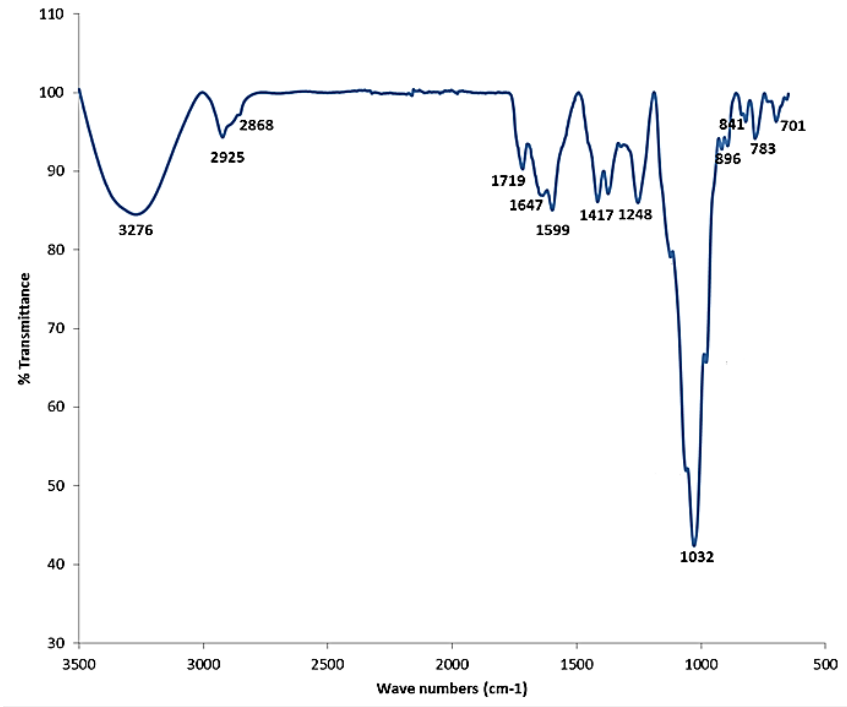


Figure Captions

Fig. 1. Intracellular Ni (a) and wall surface Ni (b) in *Paraburkholderia ultramafica* STM10279^T, *Caballeronia novacaledonica* STM10272^T and *Paraburkholderia caribensis* MWAP64^T as a function of the Ni concentration in the growing medium. Bars represent means, and error bars represent standard errors (3 replicates). Value with the same lower case letters are not significantly different at $P < 0.05$ using Fisher's LSD test.

Fig. 2. Formation of biofilm by *Paraburkholderia ultramafica* STM10279^T, *Caballeronia novacaledonica* STM10272^T and *Paraburkholderia caribensis* MWAP64^T in response to nickel stress and as a function of the exposure time 24 h (a), 48 h (b) and 72 h (c). Bars represent means, and error bars represent standard errors (6 replicates). Biofilm production occurs when the ratio is greater than two (dotted line on the graphic). Value with the same lower case letters are not significantly different at $P < 0.05$ using Fisher's LSD test.

Fig. 3. Biovolume (a), Thickness average (b) and substratum coverage (c) for *Paraburkholderia caribensis* MWAP64^T, *Caballeronia novacaledonica* STM10272^T and *Paraburkholderia ultramafica* STM10279^T with and without nickel in the growing medium. Bars represent means, and error bars represent standard errors (6 replicates). Value with the same lower case letters are not significantly different at $P < 0.05$ using Tukey's HSD test or Fisher's LSD test when data did not follow the assumptions of a parametric test.

Fig. 4. Fourier Transform Infrared (FT-IR) spectrum of the *Paraburkholderia ultramafica* STM10279^T EPS in the 500-3500 cm^{-1} range showing absorbance bands or peaks characteristic of polysaccharides.

- 1 **Table 1** Metals IC₅₀ (mM) for *Paraburkholderia ultramafica* STM10279^T, *Caballeronia novacaledonica* STM10272^T and *Paraburkholderia caribensis* MWAP64^T
 2 (Mean ± Standard Error, n = 6).

Species	IC ₅₀ (mM)							
	Ni		Co		Cr		Mn	
<i>P. caribensis</i> MWAP64 ^T	1.9	± 0.9c	1.1	± 0.1b	0.2	± 0.1b	11.1	± 0.9b
<i>P. ultramafica</i> STM10279 ^T	30.7	± 2.8a	3.9	± 0.9a	0.5	± 0.1b	32.4	± 1.6a
<i>C. novacaledonica</i> STM10272 ^T	4.8	± 0.5b	5.8	± 0.9a	1.5	± 0.3a	29.1	± 5.7a

- 3 Value with the same lower case letters within a column are not significantly different at P < 0.05 using Fisher's LSD test.

- 4 **Table 2** Metals contents in the shoots and roots of *Tetraria comosa* as a function of various treatments (mean ± SE n = 3).

Plant part	Treatment	Ni		Co		Cr		Mn	
		(mg Kg ⁻¹)		(mg Kg ⁻¹)		(mg Kg ⁻¹)		(mg Kg ⁻¹)	
Shoot	Control	132	± 18a	13.5	± 1.8a	191	± 29ab	415	± 29a
	<i>P. caribensis</i> MWAP64 ^T	159	± 18a	11.2	± 1.0a	242	± 12a	380	± 9a
	<i>P. ultramafica</i> STM10279 ^T	155	± 12a	9.4	± 1.0a	191	± 13b	388	± 19a
	<i>C. novacaledonica</i> STM10272 ^T	154	± 10a	15.2	± 0.5a	227	± 16ab	468	± 7a
Root	Control	1377	± 111a	284	± 26a	2168	± 29a	3351	± 232a
	<i>P. caribensis</i> MWAP64 ^T	582	± 54c	126	± 13b	811	± 12b	1806	± 150c
	<i>P. ultramafica</i> STM10279 ^T	766	± 68bc	155	± 13b	1158	± 13b	2097	± 159b
	<i>C. novacaledonica</i> STM10272 ^T	966	± 81b	206	± 19ab	1414	± 16b	2698	± 165ab

- 5 Value with the same lower case letters within a column for each plant part are not significantly different at P < 0.05 using Tukey's HSD test or Fisher's LSD test

- 6 when data did not follow the assumptions of a parametric test.