

Recovery of ultramafic soil functions and plant communities along an age-gradient of the actinorhizal tree Ceuthostoma terminale (Casuarinaceae) in Sabah (Malaysia)

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Celestino Quintela-Sabaris, Etienne Auber, Sukaibin Sumail, Jean-Francois Masfaraud, Michel-Pierre Faucon, et al.. Recovery of ultramafic soil functions and plant communities along an age-gradient of the actinorhizal tree Ceuthostoma terminale (Casuarinaceae) in Sabah (Malaysia). Plant and Soil, 2019, 440 (1-2), pp.201-218. 10.1007/s11104-019-04085-y. hal-02376402

HAL Id: hal-02376402 https://hal.science/hal-02376402

Submitted on 22 Nov 2019

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1	Recovery of Ultramafic Soil Functions and Plant Communities along
2	an Age-gradient of the Actinorhizal Tree Ceuthostoma terminale
3	(Casuarinaceae) in Sabah (Malaysia)
4	
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23	Keywords: Nitrogen, Pioneer plants, Plant-soil interaction, Soil enzymes, Soil recovery, Tropical

24 areas

25

26 Abstract

Background and aims Pioneer plants may improve the ecological restoration of degraded ultramafic areas by plant-soil interaction processes. In this study, we assess the effect of the pioneer actinorhizal tree *C. terminale* (Casuarinaceae) on the recovery of plant communities and soil functions on degraded tropical ultramafic sites.

31

32 *Methods* Soil and plant samples were collected along a tree-age gradient in two degraded ultramafic 33 sites in Sabah (Northern Borneo, Malaysia): a Technosol and a Leptosol. Chemical composition of 34 plants and soils, and biological activity of soils were assessed at both sites. Plant colonisation was 35 assessed by plot vegetation surveys.

36

Results An improvement in soil fertility parameters (pH reduction from 8.5 to 6.8, an increase in the
concentrations of several nutrients and enhanced soil enzyme activities) was observed along the *C*. *terminale* age gradient. However, plant cover and diversity was only improved around mature trees
at the site that was not impacted by mining.

41

42 *Conclusion C. terminale* promotes the recovery of several soil functions, mainly related to the
43 storage and recycling of N, P, K, S. Besides plant-soil feedback, other environmental factors (i.e.
44 exposition to sunlight, drought) may play an important role on revegetation of ultramafic soils.

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- 46
- 47

48 Introduction

49 Ultramafic rocks are fragments of oceanic lithosphere or derivation of upper mantle materials that 50 are locally abundant along tectonic plate margins (Echevarria 2018). Ultramafic bedrock consisting 51 of the magnesium-iron-silicate peridotite can be altered by low-temperature metamorphism of 52 peridotite ('serpentinization') leading to the formation of serpentine-group minerals (i.e. antigorite, 53 chrysotile, lizardite) (Alexander 2004; Van der Ent et al. 2018).

54

55 Due to its chemical (richness in metals such as Ni, Cr or Co) and mineralogical properties (more 56 than 90% of dark-colored mafic minerals such as olivine), ultramafic rocks have been mined for 57 metal extraction or exploited for use as ornamental stones, road-base aggregate or railway track 58 ballast (Guillot and Hattori 2013; Mudd and Jowitt 2014). Ultramafic rock-derived soils are also important areas for plant biodiversity, hosting a numerous endemic and threatened species (Van derEnt et al. 2015a).

61

62 As a major component of terrestrial ecosystems, soils generate a range of important functions, such 63 as biodiversity provisioning or storage, filtration, and transformation of substances (Adhikari and 64 Hartemink 2016). Surface operations on ultramafic areas, such as mining or quarrying, degrade soil 65 conditions by compaction and loss of topsoil, reducing the soil capacities to render these functions 66 (Echevarria and Morel 2015). Moreover, non-stabilised ultramafic materials may constitute a source 67 of air and water pollution, due to wind-borne dispersal of dust particles with asbestos and metals or 68 leaching of pollutants to groundwater (O'Dell and Claassen 2009; Raous et al. 2010). Thus, 69 revegetation of degraded ultramafic areas has several environmental benefits (Tordoff et al. 2000). 70 However, restoration of ultramafic degraded sites is a complicated task that must address several 71 typical characteristics of the material which severely limit the establishment of plants; *i.e.* low 72 concentrations of essential nutrients, unbalanced calcium (Ca) and magnesium (Mg) concentrations, 73 presence of potentially toxic concentrations of metals -mainly Ni and chromium (Cr)-, (Brady et al., 74 2005; Kazakou et al., 2008); as well as other properties related to soil fertility such as low cation 75 exchange capacity (CEC) or low water holding capacity (WHC) (Bradshaw 1997; O'Dell and 76 Claassen 2009). These harsh conditions may reach extreme values in serpentinite-derived soils in 77 tropical areas: high total and exchangeable Mg, very high Mg:Ca molar quotients, low 78 exchangeable K (down to 0.05 cmol⁽⁺⁾ kg⁻¹), and circum-neutral pH at surface (pH 7), and highly 79 alkaline at depth (up to pH 9.8) (Van der Ent et al. 2018). 80

81

In general, revegetation of human-degraded areas requires the recovery of a soil nutrient stock (nitrogen (N), phosphorus (P), potassium (K)) and organic matter sufficient to sustain ecosystem functions (Bradshaw 1997; Wong 2003). This nutrient stock can be built up by the application of stored topsoil or by the use of organic amendments (Tordoff et al. 2000; O'Dell and Claassen 2009). Pioneer species native to ultramafic substrates may be a feasible alternative for the revegetation of degraded areas, as they are already adapted to the particular conditions of local ultramafic soils (Whiting et al. 2004; Van der Ent et al. 2013).

89

90 Pioneer plants can ameliorate soil conditions as they are able to extract essential nutrients, such as 91 Ca, P, K, from soils, accumulate them in their biomass and then transfer them to the soil in litter 92 organic matter (Bradshaw 1997, 2000). Nitrogen is stored in soils mainly in organic matter, 93 although in some areas N-rich bedrock is an overlooked nitrogen source (Morford et al. 2011).

94 Thus, in post-operation scenarios, where organic topsoil is usually lacking, fixation of atmospheric 95 nitrogen (N_2) by legumes or actinorhizal plants is usually the only source of this essential nutrient in 96 the ecosystems (Bradshaw 1997, 2000). Improvement of soil conditions by pioneer species has been 97 proposed as one of the main theories explaining ecological succession (i.e. facilitation, Connell and 98 Slatyer 1977). Thus, pioneer plants alleviate extreme soil conditions, making soil less suitable to 99 themselves and more suitable to later successional species, which then colonise (Pulsford et al. 100 2016). Facilitation processes, which can be mediated by soil-plant interactions or other factors such 101 as protection from excessive sunlight, have been important for ecological succession on extreme 102 environments, such as mine tailings or ultramafic areas (McCoy et al. 1999; Yang et al. 2015).

103

South and Southeast Asia host the largest ultramafic areas in the world (Galey et al. 2017). Mining activities for Ni have a major impact on the biodiversity of ultramafic ecosystems in some countries of this region, such as Indonesia (Van der Ent et al. 2013). In Sabah (Malaysia, Northern Borneo), ultramafic soils occupy an area 3,500 km², with the area around Mount Kinabalu hosting several substantial outcrops (Van der Ent et al. 2015b). Serpentinite-derived Hypermagnesic Leptosols around Mount Kinabalu typically have highly distinct plant communities, the so-called 'Pioneer Casuarina scrub' and 'Mature Mixed Casuarina forest' (Van der Ent et al. 2016).

111

112 Ceuthostoma terminale L.A.S. Johnson (Casuarinaceae), is a dioecious pioneer tree species 113 distributed from Borneo to New Guinea, where it occupies ridges and slopes on ultramafic soils, where the rainforest canopy is interrupted (Johnson 1988) (Fig. 1A). C. terminale (and other 114 115 Casuarinaceae, including *Gymnostoma nobile* and *G. sumatranum*) are the dominant species on serpentinite-soils in Sabah (Van der Ent et al. 2016). As other Casuarinaceae, C. terminale leaves 116 117 are reduced to small scales, a plant strategy to deal with low soil fertility (Dörken and Parsons 118 2017). Thus, photosynthetic function is performed by the deciduous green branchlets (Fig. 1B). The 119 Casuarinaceae exhibit actinorhizal plant species, which host nitrogen-fixing actinobacteria (e.g. 120 Frankia) that facilitate N-assimilation by the plant (Benson and Dawson 2007). Root nodules have 121 been found on *C. terminale*, although the specific *Frankia*-strain has not yet been identified (Fig. 122 1C).

123

Actinorhizal plants have shown an important role in primary succession (e.g. ecosystem development on moraines left by retreating glaciers, Bradshaw 2000), and are useful for the recovery of marginal soils and degraded mine areas (Valdés 2007; Sheoran et al. 2010). Most actinorhizal plants occur in boreal and temperate ecosystems in both hemispheres, and family Casuarinaceae is the only that grows in tropical and subtropical areas (Dawson 2007). *Casuarina* species (mainly *Casuarina equisetifolia*) have been widely used for the revegetation of extremely degraded soils in tropical areas of America, Africa and Asia (Valdés 2007; Diagne et al. 2013). *C. terminale* has readily colonised ultramafic Technosols in an abandoned serpentinite quarry South of Kinabalu Park (Sabah, Malaysia) (C. Quintela-Sabarís, *personal observation*). At that site, *C. terminale* plants create litter layers that are colonised by different plant species. Thus, *C. terminale* may be an interesting species for the restoration of tropical degraded ultramafic areas.

135

We hypothesise that *C. terminale* locally alleviates harsh conditions on ultramafic soils by root and litter effects on soil properties, thereby facilitating the colonisation by other plant species. To test this hypothesis, we investigated two ultramafic sites where *C. terminale* is present: (i) a valley subjected to recurrent landslides, and; (ii) a quarry where the soils have been human-degraded. In order to assess possible plant-soil feedback effects by *C. terminale*, we studied the development of soil chemical and biological properties along an age-gradient of *C. terminale* plants and compared the herbaceous communities at each site.

143

144 Material and Methods

- 145
- 146 Studied sites

Two ultramafic areas from Sabah (Northern Borneo, Malaysia) were included in this study: (i) an 147 old serpentine quarry near Bukit Kulung (hereafter "Kulung"), and; (ii) an ultramafic area in the 148 valley of the Wuluh River (hereafter "Wuluh") (Fig. 2A). At both sites, C. terminale is a dominant 149 150 species in the local landscape. Quarrying at the Kulung site was related to Mamut Copper Mine 151 (MCM), which was abandoned about 20 years ago, when mining activities ceased (van der Ent and 152 Edraki 2018). Currently, this area is included in the Bukit Hampuan Forest Reserve. The total 153 surface of the quarry is about 1.5 ha. The soil is a Spolic Technosol composed of a shallow layer of 154 serpentinite tailings on top of serpentinite bedrock. Hundreds of *C. terminale* plants are growing in 155 the guarry, and several mature trees (up to 15 m tall) were also found (Fig. 2B). Around of each *C*. 156 terminale plant, litter (composed of deciduous photosynthetic branchlets) accumulates below plant 157 canopies. This layer may reach 40 cm thickness under mature trees.

Wuluh area is one of the three major serpentinite occurrences in Kinabalu Park (Van der Ent et al. 2018). The sampled site consists of several serpentinite landslides of different ages, from recent landslides without vegetation (area around 0.5 ha) to old landslides covered by a *C. terminale* dominated forest (Fig. 2C). The soils in recent landslides are Hypereutric (Hypermagnesic) Leptosols composed mainly of fine degraded serpentinite debris. In the old landslides, the soil is

163 covered by a continuous thick layer of *C. terminale* branchlets (O Horizon), with a well-developed

- 164 A-horizon (Van der Ent et al. 2018).
- 165
- 166 Collection of samples

167 At each site, a stratified sampling, following an age gradient of *C. terminale* plants, was undertaken. 168 Nine (at Wuluh site) or ten (at Kulung site) non-vegetated (NV) soil samples were collected from sampling points separated at least 2m from any vascular plant. Then, five plants from each of the 169 170 following three age-classes (we used plant height as a proxy for age) were selected: saplings (S, height < 1 m), young trees (YT, 1 m <height < 5 m) and mature trees (MT, height > 10 m, but in 171 172 most cases around 15 m height), totaling 24-25 soils and 15 plants sampled per site. Soil samples 173 were collected beneath the canopies of the selected plants. All soil samples were collected from the 174 A-layer at 0-20 cm depth in both sites.; due to soil structure we were unable to sample deeper soil at 175 Kulung (it was actually the serpentinite bedrock) and soils at Wuluh were sampled at the same 176 depth to better compare soil evolution between sites. Litter samples (each composed by a 10×15 177 cm ziplock plastic bag full of litter) were collected if present. A sample of green branchlets was 178 taken from the canopy of each selected plant. In order to investigate variation in soil structure near 179 *C. terminale* plants, blocks of undisturbed soil were also collected from the superficial soil layer 180 (excluding litter) using Kubiena boxes (9×6×5 cm) in three points (non-vegetated soil, soil close to 181 a young tree and soil close to a mature tree). Kubiena boxes (Jongerius and Heintzberger 1963) with 182 two open faces were slowly inserted into a ledge excavated in the soil. This was only possible in 183 Wuluh, because the rocky nature of Technosol at Kulung prevented the insertion of the boxes. After 184 extraction of the soil with a knife, the boxes were wrapped, secured against any disturbance in a 185 container and transported to the laboratory.

186 Finally, in order to assess the effect of *C. terminale* litter layer on plant colonisation, several 187 botanical surveys were setup at each site. The surface of the surveys varied to adapt to local 188 conditions (e.g. tree density). In Kulung, eight mature trees (MT) were selected and 16 plots of 4 imes189 4 m were delimited: eight on the area covered by litter layer around each tree and eight on NV soil 190 next to each MT plot. In Wuluh, 15 sampling plots were delimited around each of the sampled trees. 191 The surface of the plots was 1×1 m for S and YT categories, and 2×2 for MT. Plant species 192 (including ferns, grasses, forbs and woody seedlings) growing in each square were identified and 193 the cover of each species in each plot was registered using Braun-Blanquet cover classes (Braun-194 Blanquet 1964).

195

196 Analysis of soil and plant samples

197 Fresh soil samples were passed by a 5-mm sieve upon sampling, kept in plastic ziploc bags and 198 stored for 6 to 8 weeks at 4°C until analyses. The activity of soil enzymes alkaline phosphatase, 199 arylsulfatase, β -glucosidase and urease, and the degradation of Fluorescein Diacetate (FDA) were 200 determined in soil fresh subsamples.

Urease activity was determined using the colorimetric method described by (Kandeler and Gerber 1988). Alkaline phosphatase, ß-glucosidase and arylsulfatase activities were determined using *p*nitrophenol (*p*NP) linked substrates, according to the modified method of Dick et al. (2013). Finally, fluorescein diacetate (FDA) hydrolytic activity of the soil was measured colorimetrically following the method of (Adam and Duncan 2001).

206

207 Soil subsamples were air-dried and passed through a 2-mm sieve. Soil pH was measured in H₂O, 208 after 60 min shaking, using a 1:5 (v/v) ratio. Cation Exchange Capacity (CEC) was determined 209 colorimetrically after treatment of the soil with a solution of cobalt hexamine trichloride 0.05 N, 210 following the standard method AFNOR NF-X 31-130. Exchangeable concentrations of Ca⁺², Mg⁺² 211 and K⁺ in the soil were determined on the filtered soil-cobalt hexamine extracts by ICP-AES 212 (Liberty II, Varian). Total soil C and N was estimated by combustion in a CHNS analyser (Vario 213 Micro Cube, Elementar, Germany). Soil available phosphorus (Olsen-P) was extracted with a 214 solution of NaHCO₃ and quantified by reaction with ascorbic acid, following the standard method 215 AFNOR NF ISO 11263. Soil nickel availability was evaluated after extraction with DTPA-TEA (0.005 M DTPA with 0.01 M CaCl₂ and 0.1 M triethanolamine (TEA) at pH 7.3, 1:2 w/v, 2h 216 shaking) (Lindsay and Norvell 1978). Soil subsamples were ground in a ceramic mortar and 0.5 g of 217 218 dry ground soil were digested in 2 mL of concentrated HNO₃ and 6 mL of concentrated HCl on a 219 hot plate at 105°C. The final solutions were filtered (0.45 μm DigiFILTER, SCP science, Canada) 220 and brought to volume (50 mL) with deionised water. Pseudo-total soil concentrations of Ca, Co, 221 Cr, Fe, K, Mg, Mn, Ni, P and S were determined by analysis of the digests with ICP-AES (Liberty 222 II, Varian).

223 The development of soil structure was observed in soil thin sections. These sections (9 \times 6 cm, 20 224 µm-thick) were prepared according to the method described by Jongerius and Heintzberger (1975). 225 In brief, the soil samples were carefully extracted from Kubiena boxes and dried by replacing water 226 by acetone. Then, the samples were impregnated under vacuum with polyester resin (EPON 812, 227 Shell). The consolidated bloc was cut in two equivalent parts. One part was fixed on a thin glass 228 with the same size (9 \times 6 cm) and sliced until having a soil section of 20 μ m. The thin sections 229 were scanned using a flatbed scanner (EPSON V750 Perfection Pro) at 1200 dpi. Magnified images 230 were obtained with a Leica stereomicroscope MZ FLIII microscope.

Green branchlets were cleaned with tap water and rinsed with deionised water. Litter samples and cleaned branchlets were dried (60°C) until constant weight. Dry material was finely ground using a ball mill. Subsamples (0.5 g) of dry and ground tissues were digested at 95°C in 2.5 mL of concentrated HNO₃ and 5 mL of H_2O_2 (30%). The final solutions were filtered (0.45 µm DigiFILTER) and completed up to 25 mL with deionised water. Ca, Co, Cr, K, Mg, Ni and P concentrations were measured by ICP-AES (Liberty II, Varian). C and N were quantified in dry ground samples using a CHNS analyzer (Vario Micro Cube, Elementar, Germany).

- 239
- 240 Data analysis

241 The variation in soil properties along *C. terminale* age-gradient was assessed for each site (Wuluh

- and Kulung) using a Principal Component Analysis (PCA). Differences in soil variables (pH,
 concentrations of C, N, P, K and Ni-DTPA, CEC and FDA degradation) were further explored by
 two-way ANOVAs, with the factors *site* (Wuluh *vs*. Kulung) and *age-class* (non-vegetated, saplings,
 young trees and mature trees). The data were log-transformed to fit ANOVA assumptions.
- Variation in concentrations of N, P, K and Ca in branchlets was assessed by blocked two-way ANOVA (factors *site* and *age-class*). The differences within each site for these variables were analyzed by separated one-way ANOVAs (factor *age-class*) followed by Tukey post-hoc analyses. Information on development of soil structure provided by soil-thin sections was used to complement soil chemical and biological data.
- 251 Differences in plant species composition between litter cover classes were assessed with 2D Non-252 Metric Multidimensional Scaling (NMDS). NMDS were computed separately for each site (Wuluh 253 or Kulung). Moreover, plant species diversity in vegetation surveys was calculated using Shannon's 254 diversity index (H'). This index was computed with the following formula: H' = $-\Sigma p_i \ln(p_i)$, where *i* 255 corresponds to each species in the survey and p_i is the proportional abundance of species *i*. Species' 256 cover (assessed as Braun-Blanquet cover classes) was used as a proxy for the abundance of each 257 species. H' was computed with function *diversity*, and NMDS was computed using function 258 *metaMDS*, both of them from package *vegan* for R (Oksanen et al. 2017). Differences in species 259 diversity were assessed separately for each site using one-way ANOVAs. PCAs and ANOVAs were 260 performed in SPSS (v. 15, SPSS Inc., Chicago, IL, USA).
- 261

262 Results

263 Variation in soil properties

The non-vegetated (NV) soils from Kulung and Wuluh had extreme chemical properties characteristic of ultramafic soils (table 1): high pH, extremely low concentration of essential nutrients N, P and K, extremely high concentrations of Mg, low CEC (with dominance of Mg²⁺ in

the exchange complex) and low biological activity. The conditions in the NV soils from Wuluh were harsher than at Kulung as shown by pseudo-total Ca, K and P and exchangeable Ca concentrations, which were lower than instrumental detection limit.

270 Principal components analyses (PCA) were performed using all soil samples from each site. The 271 PCA performed on samples from Kulung (Technosol) identified six principal components (PC) that 272 explained 87% of total variance. pH was negatively correlated to PC1 (28% of variance), whereas 273 CEC, variables related to organic matter (C, N) and biological activity (Alkaline-phosphatase, β-274 glucosidase) had positive contributions on PC1. The pseudo-total concentrations of K, P and S had 275 positive loadings on PC2 (16% variance), whereas arylsulfatase activity and FDA hydrolysis were 276 positively correlated to both PCs. The projection of soil samples on the space determined by PCs 277 showed a gradient from NV to mature tree (MT) soil samples along PC1 (Fig 3A). This indicated an 278 improvement of parameters of soil chemical and biological fertility along *C. terminale* age classes 279 (i.e. pH reduction, increase in CEC and exchangeable K and Mg, increase in C and N, increase in 280 several microbial activities in the soil), but also an increase in available Ni. It is interesting to note 281 that during soil sampling at Kulung we found cluster roots in a C. terminale sapling. Cluster roots 282 are bottle brush-like structures in roots with dense packing of root hairs.

283 In samples from Wuluh site (Leptosol) four PCs, explaining 92 % of variance, were determined. 284 The first PC (54% of variance) had negative contributions by pH, as occurred in Kulung, and by pseudo-total Mg, Ni and Cr; whereas CEC, pseudo-total concentrations of C, N, P, K, S, 285 exchangeable K, P-Olsen and all microbial enzyme activities were positively correlated to that PC. 286 287 Pseudo-total concentrations of metals Co, Fe, Mn, Cr and Ni had positive contributions on PC2 288 (20% of variance). As was observed for Kulung, the soil samples in Wuluh were also arranged along an increasing fertility gradient in PC1 from NV samples (in the most negative positions of 289 290 PC1) to MT (Fig 3B). Soil samples from saplings (S) had also positive values on PC2, which 291 indicated an increase in pseudo-total concentrations of Mn, Co and Cr on those samples.

292 ANOVAs performed on ten selected variables, related to soil chemical fertility on ultramafic soils 293 (pH, CEC, concentrations of C, N, pseudo-total Ca and Mg, Olsen P, exchangeable K and DTPA-294 extractable Ni, and FDA degradation activity) showed an improvement of soil conditions along age-295 gradient (i.e. factor age-class had significant effects for the ten variables) (Table 2, Fig. 4). For 296 example, N concentrations increased from 0.01 and 0.03 % in NV soil in Wuluh and Kulung to 0.36 297 and 0.12% in soil around mature trees in those sites. In all variables (except C, Mg and FDA 298 degradation) there were significant interactions between site and age-class (i.e. responses along age-299 gradient were different in each site). However, in most cases these parameters showed the same 300 trend of variation –decrease (pH) or increase (CEC, N, K, etc.) along time- in the two sites, and the 301 significant interaction was due to the extremely high values of MT samples from Wuluh. Those 302 samples were collected from the A-horizon below a thick organic horizon (in some places more
303 than 60 cm thick), and two samples were excluded from the ANOVAs due to extremely high C
304 concentrations (above 30%, thus representing an O-horizon).

305 Complementary to information provided by chemical analyses, microscopic observation of soil thin 306 layers taken from the Wuluh site revealed a distinct gradient of increased soil structure and organic 307 matter enrichment from NV to MT soils (Fig. 5 and supplementary material figures 1 to 3). The 308 profile in NV soil was essentially mineral (Fig. 5 A,D), without any apparent aggregated structure. 309 Macro porosity occurred around thicker elements and vertical pores were also present. Meso and 310 micro porosity were not evident, and were created as a function of the arrangement of mineral 311 elements. Colour changes in the sample from YT (Fig. 5 B,E) suggested an enrichment in organic 312 matter, and structure was observed. Meso porosity was more important and the first roots were 313 present. The MT soil, collected below litter layer (Fig. 5 C,F) had a beige color that suggested the 314 abundance of fine organic matter associated with mineral elements in aggregates. Roots were 315 abundant and the structure gave the soil a higher porosity than in previous samples. The frequent 316 observation of roots and faecal pellets was suggestive of abundant biological activity.

317

318 Variation in branchlets and litter composition

319 Despite very low concentrations of essential nutrients in the soil, C. terminale was able to 320 accumulate high concentrations of N, P, K, S and Ca (overall means of 1.1 % and 283, 3819, 616 321 and 7645 mg kg⁻¹, respectively) in the photosynthetic branchlets (table 2). Moreover, the average 322 Ca:Mg molar ratio in branchlets was close to two, and the metal concentrations were quite low (e.g. 323 Fe and Ni are 42 mg kg⁻¹ and 14.7 mg kg⁻¹, respectively). The analysis of fresh (i.e. not degraded) litter samples showed that for several nutrients (P, K, Na) concentrations in litter have been 324 325 drastically reduced to values around 10% of those in the branchlets. In contrast, other elements were 326 slightly reduced (e.g. C, N, S) or even increased their concentration (e.g. Fe, Mn, Ni) in litter (table 327 3).

ANOVA analyses conducted to assess possible differences between sites and age-classes for concentrations of N, P, K and Ca in branchlets of *C. terminale* showed that only K concentrations increased with age of the plants (Table 4, Fig. 6). This trend of variation was similar in both sites, and K concentration in plants from Wuluh was always higher than plants from Kulung. N and P concentrations did not varied significantly for any of the factors. The Ca concentrations in plants from Kulung were higher than plants from Wuluh, and these differences were more marked in S and YT than in MT (Fig. 6).

335

336 Effects of litter cover on plant communities

337 NMDS analyses revealed differences in plant community composition related to *C. terminale* litter 338 cover at both sites. In Wuluh site (2D stress = 0.18) plant communities in sampling points were 339 grouped according to tree age classes. Major differences were found among age-classes and only 340 small overlaps occurred between plant communities around S and YT, whereas communities around 341 mature trees were clearly separated (Fig. 7A). This is congruent with a higher cover of several shrub 342 and tree species (e.g. Macaranga kinabaluensis -MAKI-, Euphorbiaceae; Rapanea sp. -RASP-, 343 Myrsinaceae) as well as pitcher plants (Nepenthes sp. -NESP-, Nepenthaceae) that were absent 344 around YT and S. The species diversity increased with *C. terminale* age ($F_{2,12} = 9.871$, p = 0.003). Tukey post-hoc test showed that S (H' = 0.70) had lower diversity than YT (H' = 1.30, p = 0.031) 345 346 and MT (H' = 1.56, p = 0.003), whereas no difference was found between YT and MT. 347

348 In the case of Kulung, the space occupied in NMDS plot by plant communities growing on litter 349 layers around MT greatly overlapped the space occupied by communities from NV Technosol (i.e. 350 low differences in species composition and cover) (Fig. 7B). The most important differences could 351 be related to the increased cover of the sedge *Fimbristylis* sp. –FISP– (Cyperaceae, also present in all sampled plots in Wuluh river) on litter layers, as well as the presence of different shrubs and 352 353 trees (genera Macaranga and Melastoma), absent on NV soil. Moreover, no differences in diversity occurred between plant communities growing on MT (H' = 1.15) and NV plots (H' = 1.00) ($F_{1.14}$ = 354 355 0.577, p = 0.460).

356

357 Discussion

Landscape and soil degradation through mining and quarrying activities further exacerbates the extreme conditions of ultramafic soils (O'Dell and Claassen 2009). This study shows that nonvegetated Technosols derived from serpentinite bedrock in Sabah have extremely low concentrations of the essential nutrients N, P, K, S as well as reduced soil biological activity. These conditions are similar to non-vegetated Leptosols where the topsoil, which contains almost all soil nutrients and biota, was lost due to naturally-occurring landslides.

C. terminale is successful in its ability to colonise such harsh environments and to develop until mature individuals more than 15 m height, in spite of extreme conditions in the serpentinite-derived soils. The concentrations of N (1.1%), P (0.03%) and K (3.8%) in *C. terminale* branchlets are remarkably high considering the very low availability of these elements in soils. Two morphophysiological traits are responsible for these relatively high nutrient values: i) the symbiotic relationship with N-fixing *Frankia* actinobacteria, and ii) the presence of cluster roots.

370 It is known that inoculation with *Frankia* improves the nutrient status of the actinorhizal plant and 371 enhances its growth (Sayed 2011). However, the abundance of *Frankia* in soils and its nodulation 372 capacities can be limited by several biotic and abiotic factors (including soil moisture, pH, salinity 373 or the presence of toxic metals) (Diagne et al., 2013; Valdés, 2007). Thus, extreme soil conditions in 374 mine areas may limit the application of actinorhizal plants for soil restoration. The presence of 375 nodules in *C. terminale* roots from our study sites suggests that this particular *Frankia* strain is 376 highly tolerant to the prevailing ultramafic soil conditions, although isolation of the bacterium and 377 experiments in culture media will be needed to confirm this. To our knowledge, no study on the 378 identification of *Frankia* species or strains associated to *C. terminale* has been undertaken to date.

379

380 Cluster roots are considered an adaptation in nutrient-poor soils. These structures release 381 carboxylates into the rhizosphere to solubilise immobile forms of P and Fe, increasing the 382 availability and uptake of these elements (Skene 1998; Shane and Lambers 2005). There are no 383 previous references to the presence of cluster roots in the genus *Ceuthostoma*, although they have 384 been observed in several species of Casuarina, Allocasuarina and Gymnostoma (Diem et al. 2000). 385 It is known that carboxylate release is able to mobilise other micronutrients -such as Mn- (Lambers 386 et al. 2015). We hypothesise that in the case of C. terminale carboxylates may contribute to K 387 mobilisation in the soils, thus explaining the high concentrations of this element in branchlets.

388

389 C. terminale has a high P- and K- resorption efficiency. Around 90% of these elements is resorbed 390 from the branchlets before abscission. In contrast, N resorption is lower (around 38%) and elements 391 with low mobility (such as Ca) have similar concentrations in litter and in branchlets. Compared to 392 global analyses on nutrient resorption in plants (Vergutz et al. 2012), the values of P- and K-393 resorption are higher in *C. terminale* than the global average for nitrogen-fixing evergreen 394 angiosperms (P, 57.4%; K, 41.0%), whereas N resorption is quite similar to the global average for 395 nitrogen-fixers (41.8%) (Vergutz et al. 2012). These values are congruent with the different 396 availability of each element. Nitrogen is not a limiting factor to a N-fixer as *C. terminale* and thus 397 its resorption is less critical for plant success, whereas a highly conservative –P and –K strategy is 398 favoured in nutrient-poor serpentinite soils.

399 Individual trees have the ability to modify soil conditions via differential elemental uptake and 400 release of organic matter via shoots (litterfall) or roots (root exudates or root turnover) (Binkley and 401 Giardina 1998). Regarding shoots, concentrations of nutrients in senesced leaves influence 402 ecosystem nutrient cycling by affecting litter quality and litter decomposition rates (Hayes et al. 403 2014). The high C:N and N:P ratios in *C. terminale* litter are limits for its degradability and explain 404 the presence of thick litter layers around mature trees. Recalcitrant (i.e. difficult to degrade) litter is 405 a common trait in slow growing species from nutrient poor habitats (De Deyn et al. 2008). 406 Moreover, it seems to be characteristic for the Casuarinaceae family, as attested by observations in

407 Casuarina equisetifolia (Maharudrappa et al. 2000; Sayed 2011) and in Gymnostoma 408 deplancheanum (McCoy et al. 1999). Recalcitrant litter typically improves soil carbon inputs (a 409 positive effect in ultramafic soils) but tends to immobilise nutrients, depending mainly on 410 saprophytic fungi for degradation (De Deyn et al. 2008), and thus limiting the release of nutrients to 411 the soil. Nevertheless, our results show a clear effect of *C. terminale* on soil properties, with minor 412 differences between sites that can be related to differences in the starting soil conditions (e.g. 413 extremely low extractable Ca and high extractable Mg at the Wuluh site). The most important 414 changes were: (i) a marked pH reduction (that can be related to incorporation of organic matter to the soil, root exudates -especially carboxylates from cluster roots- and N₂ biological fixation (Bolan 415 416 et al. 1991)), and (ii) an increase in organic matter (indicated by higher C and N concentrations) 417 with consequent increases in CEC, exchangeable Ca and K and specially in soil biological activity. 418 The improvement of soil properties was graded along age-classes: on average, the older the plant, 419 the higher the soil fertility. For instance, FDA degradation, which has potential to broadly represent 420 soil enzyme activity and accumulated biological effects (Dick et al. 1996), showed a 10-fold 421 increase from NV to MT soils in both sites. These results are in line with findings by Izquierdo et al. 422 (2005), who observed that forestation trials with *Casuarina equisetifolia* on mined ultramafic soils 423 from Cuba yielded the highest improvements in soil biological activity, and these improvements 424 increased with the age of the plantation. Moreover, a meta-analysis on plant individual effects on 425 soil properties also indicated that stronger modifications of soil properties by plants were observed 426 in old tree plantations with light management (Waring et al. 2015).

The most positive fertility parameters were found beneath mature trees from Wuluh (i.e. the nonanthropised area). These trees (average height 20 m) were presumably older than MT from Kulung (average height 13 m). Thus, soils beneath MT from Wuluh have been subjected to *C. terminale* effects for longer time, which implied the formation of thicker litter layers and also a longer period for the degradation of the recalcitrant litter (see above) and its incorporation to soil.

432 The soils degraded by natural (e.g. landslides) or human (e.g. quarrying) impacts have reduced 433 capacities to render several functions such as water filtration or nutrient storage (Echevarria and 434 Morel 2015). Observed changes in soil properties along C. terminale age-gradient indicate the 435 recovery of several important soil functions, such as the carbon pool, nutrient storage (increased N, 436 P, K, Ca concentrations), nutrient cycling (increased enzyme activities related to cycles of C, N, P 437 and S), soil organic matter degradation (increased degradation of FDA) (Burges et al. 2016). 438 Overall, these results provide insights into the positive role of the actinorhizal plant *C. terminale* to 439 obtain functional ecosystems in degraded areas.

Plant facilitation plays an important role in the colonisation and succession and in the structurationof plant communities on ultramafic soils (McCoy et al. 1999; Oviedo et al. 2014). This facilitation

442 may occur through the improvement of chemical properties of the soil (i.e. increased nutrient 443 supply), but also through the provision of shaded sites that reduce drought stress and excessive 444 insolation on seedlings during the first few years of growth (McCoy et al. 1999; Rigg et al. 2002). 445 Colonisation and development of *C. terminale* trees on ultramafic soils have improved soil fertility 446 at both studied sites. Despite the clear changes in soil conditions, changes in plant communities 447 growing around C. terminale plants were more subtle. Along the C. terminale age-gradient we observed an increase in the cover of some shrub and tree species (Macaranga sp., Melastoma sp., 448 449 Breynia sp.) and the reduction of the cover of some herbaceous plants (Selaginella sp., Schizaea 450 spp.) and *C. terminale* (e.g. no *C. terminale* saplings were found below mature trees), although two 451 herbaceous species (the grass *Mischantus floridulus* and especially the sedge *Fimbristylis* sp.) had 452 an abundant cover in all the age classes, thus reducing the differences among plant communities. 453 Overall, a clear shift in composition of plant communities was only observed around mature trees of 454 Wuluh, whereas similar communities grow in plots on non-vegetated soil and around mature trees 455 from Kulung. Moreover, an increase in plant diversity was found only around young and matures 456 trees from Wuluh. Both results (shifts in composition or increase of biodiversity) suggest that the 457 facilitation effects on plant communities occurred only around mature trees at the Wuluh site. It was 458 hypothesised that one of the facilitation effects of *Gymnostoma deplancheanum* on ultramafic soils 459 from New Caledonia was related to shading (McCoy et al. 1999). Moreover, experiments on 460 controlled conditions showed that only plants with small seeds (~ 2 mm) were able to establish on 461 top of *G*. *deplancheanum* litter layers: small seeds were washed down the layer and stayed in humid 462 microsites whereas species with large seeds (> 6 mm) dried out on litter surface and were unable to 463 establish (McCoy et al. 1996). Our observations indicate that other environmental factors besides 464 improvement of soil chemical parameters (i.e. exposition to sunlight and drought) may have an 465 important role on the recovery of plant communities on ultramafic soils. C. terminale plants at 466 Kulung were growing isolated and fully exposed to sunlight. In this situation, surface of litter layer 467 gets dry easily, creating hostile conditions for seed germination. At Wuluh, the plant density was 468 higher and, in the case of mature trees, they formed a dense forest with a continuous litter layer. 469 Thus, we hypothesise that the higher plant diversity around mature trees at Wuluh is the result of a 470 dense tree cover at this site and increased shading of the litter layer. As a consequence, litter is kept 471 humid for a longer time favoring the germination and establishment of more plant species. 472 However, the validity of this tentative explanation should be tested under controlled conditions.

473

474 In conclusion, the establishment and growth of *C. terminale* has improved soil chemical and
475 biological parameters and important soil functions, such as nutrient conservation and cycling.
476 Moreover, a change in community structure and an improvement in the diversity of plant

477 communities was observed around mature trees growing on soil disturbed by landslides. Overall,
478 *C. terminale* appears to be a promising species for the restoration of degraded serpentinite soils in
479 SE Asia and Melanesia. However, the application of this species in ecological restoration programs
480 need the development of more research in: (i) plant reproduction, including knowledge about seed
481 viability and strategies for vegetative multiplication; (ii) agronomical practices, especially in

482 relation to planting density and management of litter layers to favor establishment of new species,

483 and, (iii) exploration of soil biota, with special emphasis on symbiotic arbuscular mycorrhiza fungi

- 484 and also saprophytic fungi able to degrade *C. terminale* litter.
- 485

486 Acknowledgements

Sabah Biodiversity Centre provided C. Quintela-Sabarís with the Access and Export Licences to develop this research in Sabah (Malaysia). Local support provided by Mr. Geofarry Gunsalam and personnel in Monggis Substation (Kinabalu Park) is warmly acknowledged. C. Quintela-Sabarís Postdoctoral contract and this research have been funded by the French National Research Agency through the national program "Investissements d'avenir" with the reference ANR-10-LABX-21-01/ LABEX RESSOURCES21, and by Region Lorraine (France). A. van der Ent is the recipient of a Discovery Early Career Researcher Award (DE160100429) from the Australian Research Council.

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Table 1 Description of non-vegetated soils from Bukit Kulung quarry (ultramafic Technosol) and Wuluh River (ultramafic Leptosol). Mean values (n = 9-10) are presented, units are indicated for each variable. P and K pseudo-total concentrations in Kulung, and S pseudo-total concentrations in both sites are below limit of quantification (K, 150 mg kg⁻¹; P, 52 mg kg⁻¹; S, 224 mg kg⁻¹), but are presented for comparative purposes.

Variable	Si	te		
_	Kulung	Wuluh		
рНн₂о	7.98	9.09		
C and N (%)				
С	0.53	0.39		
Ν	0.03	0.01		
C:N ratio	15.5	31.8		
Pseudo-total concentrations of ma	ajor and trace elen	nents (mg kg ⁻¹)		
Ca	2,580.7	< d.l.		
Fe	57 10 ³	$38.5 \ 10^3$		
Κ	72.6	< d.l.		
Mg	234.8 10 ³	$255.3 \ 10^3$		
Mn	1,297.5	633.2		
Р	11.66	< d.l.		
S	133.58	67.43		
Со	115.36	70.6		
Cr	1,222.4	948.3		
Ni	2,187.9	1,647.4		
Olsen P (mg kg ⁻¹)	1.4	1.33		
DTPA-extractable Ni (mg kg ⁻¹)	18.95	38.36		
CEC and Exchangeab	le cations (cmol _c k	(g ⁻¹)		
CEC	7.98	1.69		
Ca	1.47	0		
Mg	5.82	4.31		
K	0.02	0.01		
Ca:Mg molar quotient	0.23	0		
Soil microbial activiti	es (µg product g ⁻¹	h-1)		
Alkaline Phosphatase	13.14	0.79		
Arylsulfatase	1.76	0.07		
β-Glucosidase	34.28	31.73		
Urease	2.03	0.55		
FDA hydrolysis	0.75	3.53		

505	Table 2 Two-way ANOVAs of ten variables related to soil chemical and biological fertility in two
506	ultramafic sites (Kulung and Wuluh) and 4 age-classes (non-vegetated, sapling, young tree and
507	mature tree) (for each site, n = 9-10 in NV class and n=5 in S, YT and MT classes). Site and age-
508	class were fixed factors, all variables (except pH) were log-transformed to meet ANOVA
509	assumptions. Each line corresponds to one ANOVA. P: ***p<0.001, ** p<0.01, * p<0.05, ns = not
510	significant.

Variable	Site (df = 1)			A	Age-class (df = 3)			*Age-o (df = 3)	Error (df = 39)	
	MS	F	р	MS	F	р	MS	F	р	MS
pН	3.12	42.0	***	7.2	97.0	***	0.69	9.3	***	0.07
С	0.004	0.1	ns	2.2	45.9	***	0.1	2.7	ns	0.05
Ν	0.002	0.1	ns	2.0	55.5	***	0.3	9.4	***	0.04
P-Olsen	1.38	27.6	***	1.30	26.0	***	1.04	20.7	***	0.05
K_{Exch}	0.003	0.1	ns	1.14	37.7	***	0.20	6.7	**	0.03
CEC	0.26	15.6	***	1.14	69.4	***	0.45	27.5	***	0.02
Ca	51.12	229.6	***	3.39	15.2	***	3.76	16.9	***	0.22
Mg	0.00	0.3	ns	0.01	3.1	*	0.00	2.22	ns	0.00
FDA	4.82	70.0	***	2.50	36.3	***	0.13	1.9	ns	0.07
Ni _{dtpa}	0.002	0.0	ns	0.71	10.0	***	0.52	7.4	***	0.07

Table 3 Chemical composition of *C. terminale* photosynthetic branchlets and litter. Mean values for branchlets (n = 15) and litter (n = 9 - 15) are presented for each site and averaged for all samples. Units are indicated for each variable. P and S concentrations in litter are below limit of quantification (P, 125 mg kg⁻¹; S, 525 mg kg⁻¹), but are presented for comparative purposes.

516

Variable	Ε	Branchle	ts		Litter		Average element concentrations in plants (Kirkby 2012)		
	Kulung	Wuluh	Overall mean	Kulung	Wuluh	Overall mean			
				C and N	(%)				
С	49.8	52.0	50.9	50.2 51.4		50.6	-		
Ν	1.1	1.0	1.1	0.8	0.7	0.8	1.5		
C:N ratio	46.2	50.0	48.1	63.7 73.6 67.4			-		
	Pseu	do-total	concentrat	ions of maj	or and tra	ce element	ts (mg kg ⁻¹)		
Р	278.1	288.6	283.3	4.3	65.4	28.2	2,000		
Κ	2,600	5,037	3,819	255	316	279	10,000		
Ca	8,464	6,827	7,645	9,317	317 7,005		5,000		
Mg	2,214	4,073	3,144	1,581	4,847	2,859	2,000		
Ca:Mg quotient	2.75	1.17	1.96	4.18	1.3	3.05	-		
Fe	49	36	42	95	245	154	100		
Mn	98	75	86	119	88	107	50		
Na	912.5	722.6	817.6	65.9	77.0	70.2	-		
S	674.3	558.5	616.4	418.9	371.7	400.4	1,000		
Zn	10.0	9.9	10.0	12.3	9.1	11.0	20		
Ni	18.5	11.0	14.7	19.5	24.8	21.6	0.1		

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Table 4 Two-way ANOVAs of N, P, K and Ca concentration in branchlets of *C. terminale* of two ultramafic sites (Kulung and Wuluh) and 3 age-classes (sapling, young tree and mature tree; for each site and age-class, n = 5). Site and age-class were fixed factors. Each line corresponds to one ANOVA. P: ***p<0.001. ** p<0.05. ns = not significant.

ANOVA	. P. ~ p<0.0	01, ••	p~0.0	1, ⁻ p<0.03, I	15 - 11	OL SI	giiniCant.			
Variable	Sit (df =	Age-cl (df =	Age-class (df = 2)			Site*Age-class (df = 2)				
	MS	F	р	MS	F	р	MS	F	р	MS
Ν	0.02 0.6 ns		0.05	2.0	ns	0.04	1.7	ns	0.03	
Р	828.5	0.12	ns	10,698.2	1.5	ns	7,476.1	1.0	ns	7,147.07
K	44,545,341	21.8	***	14,491,438	7.1	**	801,580	0.4	ns	2,047,074
Ca	20,109,475	8.3	**	2,419,290	1.0	ns	8,357,718	3.5	*	2,413,940

522 FIGURE CAPTIONS

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Figure 1 A, mature *C. terminale* tree (height ~ 15 m) growing in an abandoned serpentine quarry.
B, detail of photosynthetic branchlets. The scale-like leaves are arranged in whorls (4 leaves per whorl). The yellow arrow marks one of the furrows where the stomata are hidden. C, mass of *Frankia* nodules recovered from *C. terminale* roots. Note the grey mineral particles of ultramafic soil attached to the nodule surface. Photos A and C are from C. Quintela-Sabarís, photo B from F.
Watteau.

530

Figure 2 Studied sites. **A**, regional map of SE Asia and local map (N of Borneo) showing the sampled areas (red stars in local map). Dark grey polygons mark protected areas in Sabah. **B**, detail of the abandoned serpentinite quarry of Bukit Kulung. Two young *C. terminale* trees can be seen in the right foreground, and several mature trees are seen in the background. Note the reddish litter layer below their canopies. **C**, detail of a small landslide in Wuluh river, with several young *C. terminale* trees growing in the open area. Photos from C. Quintela-Sabarís.

538 **Figure 3** PCA analysis of soil samples from Kulung **(A)** and Wuluh **(B)**. In each graph, soil samples 539 are marked with different colors according to the age-class (NV, non-vegetated; S, sapling; YT, 540 young tree; MT, mature tree). Empty dots mark the centroid for each group, with lines connecting to each of the samples (for each site, n = 9-10 in NV class and n=5 in S, YT and MT classes). For each 541 542 site, contributions of soil variables to each of the two first Principal Components (PC) are indicated 543 in the insets. Pseudo-total concentrations of elements are indicated by their chemical symbol; CN. 544 C/N ratio; *CaMq*, Ca/Mg molar ratio; *CEC*, cation exchange capacity; Ca^{2+} , K^+ , Mg^{2+} , exchangeable 545 concentrations of Ca, K and Mg; Ni_{DTPA}, DTPA-extractable Ni concentration; AP, alkaline-546 phosphatase; *ARY*, arylsulfatase; *BG*, β -glucosidase; *FDA*, FDA degradation; *UR*, urease. 547

Figure 4 Variation in selected soil properties along age-gradient of *Ceuthostoma terminale*. In each
graph, average values (± SD) in each site (blue color, Kulung; green color, Wuluh) for each ageclass (NV, non-vegetated; S, saplings; YT, young trees; MT, mature trees) are presented (for each
site, n = 9-10 in NV class and n=5 in S, YT and MT classes). Different capital and small-case letters
indicate significant differences (p < 0.05, Tukey post-hoc test) among age-classes in Kulung and
Wuluh sites, respectively.

Figure 5 Structure in soil samples from non vegetated (A, D), young tree (B, E) and mature tree (C,
F) sampling points in at the Wuluh site. Images D to F are detailed views from the white marked
areas in images A to C. D, example of thick mineral element in the middle of a finer mineral matrix,
pore with air bubbles (as artifacts) to the right. E, different root sections and yet unidentified root
structures present in pores. F, longitudinal section of a root in the middle of organo-mineral matrix,
thick mineral element to the right.

Figure 6 Variation in the concentrations of N, P, K and Ca in branchlets of *C. terminale* along an age-gradient. In each graph, average values (\pm SD) in each site (blue, Kulung; green, Wuluh) for each age-class (S, saplings; YT, young trees; MT, mature trees) are presented (n = 5 for each site and age-class). Different capital and small-case letters indicate significant differences (p < 0.05, Tukey post-hoc test) among age-classes in Kulung and Wuluh, respectively.

Figure 7 NMDS analyses of plant communities around *C. terminale* plants at Wuluh (A) and
Kulung (B) sites. Colored polygons are convex hulls connecting plant communities on bare soil
(gray), and around *C. terminale* saplings (blue), young trees (yellow) and mature trees (green) (for
each class, n = 8 at Kulung and n = 5 at Wuluh). Red letter codes indicate plant species (see
supplementary material Table S2).



575 Fig. 2:



578 Fig 3:



581 Fig 4:



584 Fig. 5:





590 Fig. 7:

