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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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**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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Keywords: Nitrogen, Pioneer plants, Plant-soil interaction, Soil enzymes, Soil recovery, Tropical
areas

25

26 **Abstract**

27 *Background and aims* Pioneer plants may improve the ecological restoration of degraded ultramafic
28 areas by plant-soil interaction processes. In this study, we assess the effect of the pioneer
29 actinorhizal tree *C. terminale* (Casuarinaceae) on the recovery of plant communities and soil
30 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

37 *Results* An improvement in soil fertility parameters (pH reduction from 8.5 to 6.8, an increase in the
38 concentrations of several nutrients and enhanced soil enzyme activities) was observed along the *C.*
39 *terminale* age gradient. However, plant cover and diversity was only improved around mature trees
40 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.

45

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

59 important areas for plant biodiversity, hosting a numerous endemic and threatened species (Van der
60 Ent 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 $0.05 \text{ cmol}^{(+)} \text{ kg}^{-1}$), 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
82 In general, revegetation of human-degraded areas requires the recovery of a soil nutrient stock
83 (nitrogen (N), phosphorus (P), potassium (K)) and organic matter sufficient to sustain ecosystem
84 functions (Bradshaw 1997; Wong 2003). This nutrient stock can be built up by the application of
85 stored topsoil or by the use of organic amendments (Tordoff et al. 2000; O'Dell and Claassen 2009).
86 Pioneer species native to ultramafic substrates may be a feasible alternative for the revegetation of
87 degraded areas, as they are already adapted to the particular conditions of local ultramafic soils
88 (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 (N₂) 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
104 South and Southeast Asia host the largest ultramafic areas in the world (Galey et al. 2017). Mining
105 activities for Ni have a major impact on the biodiversity of ultramafic ecosystems in some countries
106 of this region, such as Indonesia (Van der Ent et al. 2013). In Sabah (Malaysia, Northern Borneo),
107 ultramafic soils occupy an area 3,500 km², with the area around Mount Kinabalu hosting several
108 substantial outcrops (Van der Ent et al. 2015b). Serpentinite-derived Hypermagnesian Leptosols
109 around Mount Kinabalu typically have highly distinct plant communities, the so-called ‘Pioneer
110 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,
114 where the rainforest canopy is interrupted (Johnson 1988) (Fig. 1A). *C. terminale* (and other
115 Casuarinaceae, including *Gymnostoma nobile* and *G. sumatranum*) are the dominant species on
116 serpentinite-soils in Sabah (Van der Ent et al. 2016). As other Casuarinaceae, *C. terminale* leaves
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
124 Actinorhizal plants have shown an important role in primary succession (e.g. ecosystem
125 development on moraines left by retreating glaciers, Bradshaw 2000), and are useful for the
126 recovery of marginal soils and degraded mine areas (Valdés 2007; Sheoran et al. 2010). Most
127 actinorhizal plants occur in boreal and temperate ecosystems in both hemispheres, and family
128 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.

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.

Material and Methods

Studied sites

Two ultramafic areas from Sabah (Northern Borneo, Malaysia) were included in this study: (i) an old serpentinite quarry near Bukit Kulung (hereafter “Kulung”), and; (ii) an ultramafic area in the valley of the Wuluh River (hereafter “Wuluh”) (Fig. 2A). At both sites, *C. terminale* is a dominant species in the local landscape. Quarrying at the Kulung site was related to Mamut Copper Mine (MCM), which was abandoned about 20 years ago, when mining activities ceased (van der Ent and Edraki 2018). Currently, this area is included in the Bukit Hampuan Forest Reserve. The total surface of the quarry is about 1.5 ha. The soil is a Spolic Technosol composed of a shallow layer of serpentinite tailings on top of serpentinite bedrock. Hundreds of *C. terminale* plants are growing in the quarry, and several mature trees (up to 15 m tall) were also found (Fig. 2B). Around of each *C. terminale* plant, litter (composed of deciduous photosynthetic branchlets) accumulates below plant 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 (Hypermagnesian) Leptosols composed mainly of fine degraded serpentinite debris. In the old landslides, the soil is

covered by a continuous thick layer of *C. terminale* branchlets (O Horizon), with a well-developed A-horizon (Van der Ent et al. 2018).

Collection of samples

At each site, a stratified sampling, following an age gradient of *C. terminale* plants, was undertaken. 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 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 most cases around 15 m height), totaling 24-25 soils and 15 plants sampled per site. Soil samples were collected beneath the canopies of the selected plants. All soil samples were collected from the A-layer at 0-20 cm depth in both sites.; due to soil structure we were unable to sample deeper soil at Kulung (it was actually the serpentinite bedrock) and soils at Wuluh were sampled at the same depth to better compare soil evolution between sites. Litter samples (each composed by a 10 × 15 cm ziplock plastic bag full of litter) were collected if present. A sample of green branchlets was taken from the canopy of each selected plant. In order to investigate variation in soil structure near *C. terminale* plants, blocks of undisturbed soil were also collected from the superficial soil layer (excluding litter) using Kubiena boxes (9×6×5 cm) in three points (non-vegetated soil, soil close to a young tree and soil close to a mature tree). Kubiena boxes (Jongnerius and Heintzberger 1963) with two open faces were slowly inserted into a ledge excavated in the soil. This was only possible in Wuluh, because the rocky nature of Technosol at Kulung prevented the insertion of the boxes. After extraction of the soil with a knife, the boxes were wrapped, secured against any disturbance in a container and transported to the laboratory.

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

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.

201 Urease activity was determined using the colorimetric method described by (Kandeler and Gerber
202 1988). Alkaline phosphatase, β -glucosidase and arylsulfatase activities were determined using *p*-
203 nitrophenol (*p*NP) linked substrates, according to the modified method of Dick et al. (2013).
204 Finally, fluorescein diacetate (FDA) hydrolytic activity of the soil was measured colorimetrically
205 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
216 (0.005 M DTPA with 0.01 M CaCl₂ and 0.1 M triethanolamine (TEA) at pH 7.3, 1:2 w/v, 2h
217 shaking) (Lindsay and Norvell 1978). Soil subsamples were ground in a ceramic mortar and 0.5 g of
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 μ m 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 μ m-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.

231

232 Green branchlets were cleaned with tap water and rinsed with deionised water. Litter samples and
233 cleaned branchlets were dried (60°C) until constant weight. Dry material was finely ground using a
234 ball mill. Subsamples (0.5 g) of dry and ground tissues were digested at 95°C in 2.5 mL of
235 concentrated HNO₃ and 5 mL of H₂O₂ (30%). The final solutions were filtered (0.45 µm
236 DigiFILTER) and completed up to 25 mL with deionised water. Ca, Co, Cr, K, Mg, Ni and P
237 concentrations were measured by ICP-AES (Liberty II, Varian). C and N were quantified in dry
238 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
242 and Kulung) using a Principal Component Analysis (PCA). Differences in soil variables (pH,
243 concentrations of C, N, P, K and Ni-DTPA, CEC and FDA degradation) were further explored by
244 two-way ANOVAs, with the factors *site* (Wuluh vs. Kulung) and *age-class* (non-vegetated, saplings,
245 young trees and mature trees). The data were log-transformed to fit ANOVA assumptions.

246 Variation in concentrations of N, P, K and Ca in branchlets was assessed by blocked two-way
247 ANOVA (factors *site* and *age-class*). The differences within each site for these variables were
248 analyzed by separated one-way ANOVAs (factor *age-class*) followed by Tukey post-hoc analyses.
249 Information on development of soil structure provided by soil-thin sections was used to
250 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: $H' = -\sum p_i \ln(p_i)$, 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

264 The non-vegetated (NV) soils from Kulung and Wuluh had extreme chemical properties
265 characteristic of ultramafic soils (table 1): high pH, extremely low concentration of essential
266 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.

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

In samples from Wuluh site (Leptosol) four PCs, explaining 92 % of variance, were determined. 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, exchangeable K, P-Olsen and all microbial enzyme activities were positively correlated to that PC. Pseudo-total concentrations of metals Co, Fe, Mn, Cr and Ni had positive contributions on PC2 (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 PC1) to MT (Fig 3B). Soil samples from saplings (S) had also positive values on PC2, which indicated an increase in pseudo-total concentrations of Mn, Co and Cr on those samples.

ANOVAs performed on ten selected variables, related to soil chemical fertility on ultramafic soils (pH, CEC, concentrations of C, N, pseudo-total Ca and Mg, Olsen P, exchangeable K and DTPA-extractable Ni, and FDA degradation activity) showed an improvement of soil conditions along age-gradient (i.e. factor *age-class* had significant effects for the ten variables) (Table 2, Fig. 4). For example, N concentrations increased from 0.01 and 0.03 % in NV soil in Wuluh and Kulung to 0.36 and 0.12% in soil around mature trees in those sites. In all variables (except C, Mg and FDA degradation) there were significant interactions between site and age-class (i.e. responses along age-gradient were different in each site). However, in most cases these parameters showed the same trend of variation –decrease (pH) or increase (CEC, N, K, etc.) along time– in the two sites, and the 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)
324 litter samples showed that for several nutrients (P, K, Na) concentrations in litter have been
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).

328 ANOVA analyses conducted to assess possible differences between sites and age-classes for
329 concentrations of N, P, K and Ca in branchlets of *C. terminale* showed that only K concentrations
330 increased with age of the plants (Table 4, Fig. 6). This trend of variation was similar in both sites,
331 and K concentration in plants from Wuluh was always higher than plants from Kulung. N and P
332 concentrations did not varied significantly for any of the factors. The Ca concentrations in plants
333 from Kulung were higher than plants from Wuluh, and these differences were more marked in S and
334 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 ($F_{2,12} = 9.871$, $p = 0.003$).
 345 Tukey post-hoc test showed that S ($H' = 0.70$) had lower diversity than YT ($H' = 1.30$, $p = 0.031$)
 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
 352 all sampled plots in Wuluh river) on litter layers, as well as the presence of different shrubs and
 353 trees (genera *Macaranga* and *Melastoma*), absent on NV soil. Moreover, no differences in diversity
 354 occurred between plant communities growing on MT ($H' = 1.15$) and NV plots ($H' = 1.00$) ($F_{1,14} =$
 355 0.577 , $p = 0.460$).

356

357 Discussion

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

364 *C. terminale* is successful in its ability to colonise such harsh environments and to develop until
 365 mature individuals more than 15 m height, in spite of extreme conditions in the serpentinite-derived
 366 soils. The concentrations of N (1.1%), P (0.03%) and K (3.8%) in *C. terminale* branchlets are
 367 remarkably high considering the very low availability of these elements in soils. Two morpho-
 368 physiological traits are responsible for these relatively high nutrient values: i) the symbiotic
 369 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

capacities can be limited by several biotic and abiotic factors (including soil moisture, pH, salinity or the presence of toxic metals) (Diagne et al., 2013; Valdés, 2007). Thus, extreme soil conditions in mine areas may limit the application of actinorhizal plants for soil restoration. The presence of nodules in *C. terminale* roots from our study sites suggests that this particular *Frankia* strain is highly tolerant to the prevailing ultramafic soil conditions, although isolation of the bacterium and experiments in culture media will be needed to confirm this. To our knowledge, no study on the identification of *Frankia* species or strains associated to *C. terminale* has been undertaken to date.

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

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

Individual trees have the ability to modify soil conditions via differential elemental uptake and release of organic matter via shoots (litterfall) or roots (root exudates or root turnover) (Binkley and Giardina 1998). Regarding shoots, concentrations of nutrients in senesced leaves influence ecosystem nutrient cycling by affecting litter quality and litter decomposition rates (Hayes et al. 2014). The high C:N and N:P ratios in *C. terminale* litter are limits for its degradability and explain the presence of thick litter layers around mature trees. Recalcitrant (i.e. difficult to degrade) litter is a common trait in slow growing species from nutrient poor habitats (De Deyn et al. 2008). 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
 415 the soil, root exudates -especially carboxylates from cluster roots- and N₂ biological fixation (Bolan
 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).
 427 The most positive fertility parameters were found beneath mature trees from Wuluh (i.e. the non-
 428 anthropised area). These trees (average height 20 m) were presumably older than MT from Kulung
 429 (average height 13 m). Thus, soils beneath MT from Wuluh have been subjected to *C. terminale*
 430 effects for longer time, which implied the formation of thicker litter layers and also a longer period
 431 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) (Burgess 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.
 440 Plant facilitation plays an important role in the colonisation and succession and in the structuration
 441 of 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
 448 observed an increase in the cover of some shrub and tree species (*Macaranga* sp., *Melastoma* sp.,
 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 **Acknowledgements**

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

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498 **Table 1** Description of non-vegetated soils from Bukit Kulung quarry (ultramafic Technosol) and
499 Wuluh River (ultramafic Leptosol). Mean values (n = 9-10) are presented, units are indicated for
500 each variable. P and K pseudo-total concentrations in Kulung, and S pseudo-total concentrations in
501 both sites are below limit of quantification (K, 150 mg kg⁻¹; P, 52 mg kg⁻¹; S, 224 mg kg⁻¹), but are
502 presented for comparative purposes.
503

Variable	Site	
	Kulung	Wuluh
pH _{H₂O}	7.98	9.09
C and N (%)		
C	0.53	0.39
N	0.03	0.01
C:N ratio	15.5	31.8
Pseudo-total concentrations of major and trace elements (mg kg ⁻¹)		
Ca	2,580.7	< d.l.
Fe	57 10 ³	38.5 10 ³
K	72.6	< d.l.
Mg	234.8 10 ³	255.3 10 ³
Mn	1,297.5	633.2
P	11.66	< d.l.
S	133.58	67.43
Co	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 Exchangeable cations (cmol _c kg ⁻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 activities (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

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

Variable	Site (df = 1)			Age-class (df = 3)			Site*Age-class (df = 3)			Error (df = 39)
	MS	F	p	MS	F	p	MS	F	p	MS
pH	3.12	42.0	***	7.2	97.0	***	0.69	9.3	***	0.07
C	0.004	0.1	ns	2.2	45.9	***	0.1	2.7	ns	0.05
N	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

511

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.

Variable	Branchlets			Litter			Average element concentrations in plants (Kirkby 2012)
	Kulung	Wuluh	Overall mean	Kulung	Wuluh	Overall mean	
C and N (%)							
C	49.8	52.0	50.9	50.2	51.4	50.6	-
N	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	-
Pseudo-total concentrations of major and trace elements (mg kg ⁻¹)							
P	278.1	288.6	283.3	4.3	65.4	28.2	2,000
K	2,600	5,037	3,819	255	316	279	10,000
Ca	8,464	6,827	7,645	9,317	7,005	8,412	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

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<0.01, * p<0.05, ns = not significant.

Variable	Site (df = 1)			Age-class (df = 2)			Site*Age-class (df = 2)			Error (df = 24)
	MS	F	p	MS	F	p	MS	F	p	MS
N	0.02	0.6	ns	0.05	2.0	ns	0.04	1.7	ns	0.03
P	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

FIGURE CAPTIONS

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.

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.

Figure 3 PCA analysis of soil samples from Kulung (**A**) and Wuluh (**B**). In each graph, soil samples are marked with different colors according to the age-class (NV, non-vegetated; S, sapling; YT, 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 site, contributions of soil variables to each of the two first Principal Components (PC) are indicated in the insets. Pseudo-total concentrations of elements are indicated by their chemical symbol; *CN*, C/N ratio; *CaMg*, Ca/Mg molar ratio; *CEC*, cation exchange capacity; Ca^{2+} , K^+ , Mg^{2+} , exchangeable concentrations of Ca, K and Mg; Ni_{DTPA} , DTPA-extractable Ni concentration; *AP*, alkaline-phosphatase; *ARY*, arylsulfatase; *BG*, β -glucosidase; *FDA*, FDA degradation; *UR*, urease.

Figure 4 Variation in selected soil properties along age-gradient of *Ceuthostoma terminale*. In each graph, average values ($\pm$ SD) in each site (blue color, Kulung; green color, Wuluh) for each age-class (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).

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