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

Celestino Quintela-Sabaris, Etienne Auber, Sukaibin Sumail, Jean-Francois Masfaraud, Michel-Pierre Faucon, Françoise Watteau, Ramez Saad, Antony van Der Ent, Rimi Repin, John Sugau, et al.

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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)**

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5   **Celestino Quintela-Sabarís[1]*, Etienne Auber[1], Sukaibin Sumail[2], Jean-François Masfaraud[3],**

6   **Michel-Pierre Faucon[4], Françoise Watteau[1], Ramez F. Saad[1], Antony Van der Ent[1,5], Rimi**

7   **Repin[2], John Sugau[6], Reuben Nilus[6], Guillaume Echevarria[1], Sophie Leguedois[1]**

8

9   [1]Laboratoire Sols et Environnement (UMR 1120 INRA- Université de Lorraine), Vandoeuvre les

10   Nancy, France

11   [2]Sabah Parks, Kota Kinabalu, Sabah, Malaysia

12   [3]Université de Lorraine, CNRS, LIEC, F-57000 Metz, France

13   [4]AGHYLE, Agroécologie, Hydrogéochimie, Milieux et Ressources (UP.2018.C101, SFR Condorcet

14   FR CNRS 3417), Institut Polytechnique UniLaSalle, Beauvais, France

15   [5]Centre for Mined Land Rehabilitation, Sustainable Minerals Institute, The University of

16   Queensland, Queensland, Australia

17   [6]Forest Research Centre, Sabah Forestry Department, Sandakan, Sabah, Malaysia

18

19   *** Correspondence:**

20   Dr. Celestino Quintela-Sabarís

21   tino.quintela.sabaris@gmail.com

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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.

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

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.

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

Introduction

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

Due to its chemical (richness in metals such as Ni, Cr or Co) and mineralogical properties (more than 90% of dark-colored mafic minerals such as olivine), ultramafic rocks have been mined for metal extraction or exploited for use as ornamental stones, road-base aggregate or railway track ballast (Guillot and Hattori 2013; Mudd and Jowitt 2014). Ultramafic rock-derived soils are also

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59   important areas for plant biodiversity, hosting a numerous endemic and threatened species (Van der

60   Ent et al. 2015a).

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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 $\text{cmol}^{(+)}$ 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).

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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).

3

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

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 km^2, with the area around Mount Kinabalu hosting several
108 substantial outcrops (Van der Ent et al. 2015b). Serpentinite-derived Hypermagnesic 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*

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129 species (mainly *Casuarina equisetifolia*) have been widely used for the revegetation of extremely
130 degraded soils in tropical areas of America, Africa and Asia (Valdés 2007; Diagne et al. 2013). *C.*
131 *terminale* has readily colonised ultramafic Technosols in an abandoned serpentinite quarry South of
132 Kinabalu Park (Sabah, Malaysia) (C. Quintela-Sabarís, *personal observation*). At that site, *C.*
133 *terminale* plants create litter layers that are colonised by different plant species. Thus, *C. terminale*
134 may be an interesting species for the restoration of tropical degraded ultramafic areas.
135
136 We hypothesise that *C. terminale* locally alleviates harsh conditions on ultramafic soils by root and
137 litter effects on soil properties, thereby facilitating the colonisation by other plant species. To test
138 this hypothesis, we investigated two ultramafic sites where *C. terminale* is present: (i) a valley
139 subjected to recurrent landslides, and; (ii) a quarry where the soils have been human-degraded. In
140 order to assess possible plant-soil feedback effects by *C. terminale*, we studied the development of
141 soil chemical and biological properties along an age-gradient of *C. terminale* plants and compared
142 the herbaceous communities at each site.
143
144 **Material and Methods**
145
146 Studied sites
147 Two ultramafic areas from Sabah (Northern Borneo, Malaysia) were included in this study: (i) an
148 old serpentine quarry near Bukit Kulung (hereafter "Kulung"), and; (ii) an ultramafic area in the
149 valley of the Wuluh River (hereafter "Wuluh") (Fig. 2A). At both sites, *C. terminale* is a dominant
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 quarry, 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.
158 Wuluh area is one of the three major serpentinite occurrences in Kinabalu Park (Van der Ent et al.
159 2018). The sampled site consists of several serpentinite landslides of different ages, from recent
160 landslides without vegetation (area around 0.5 ha) to old landslides covered by a *C. terminale*
161 dominated forest (Fig. 2C). The soils in recent landslides are Hypereutric (Hypermagnesic)
162 Leptosols composed mainly of fine degraded serpentinite debris. In the old landslides, the soil is

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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

169   sampling points separated at least 2m from any vascular plant. Then, five plants from each of the

170   following three age-classes (we used plant height as a proxy for age) were selected: *saplings* (S,

171   height < 1 m), *young trees* (YT, 1 m <height < 5 m) and *mature trees* (MT, height > 10 m, but in

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 $4 \times$

189   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

6

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

Soil subsamples were air-dried and passed through a 2-mm sieve. Soil pH was measured in H_2O, after 60 min shaking, using a 1:5 (v/v) ratio. Cation Exchange Capacity (CEC) was determined colorimetrically after treatment of the soil with a solution of cobalt hexamine trichloride 0.05 N, following the standard method AFNOR NF-X 31-130. Exchangeable concentrations of Ca^{+2}, Mg^{+2} and K^+ in the soil were determined on the filtered soil-cobalt hexamine extracts by ICP-AES (Liberty II, Varian). Total soil C and N was estimated by combustion in a CHNS analyser (Vario Micro Cube, Elementar, Germany). Soil available phosphorus (Olsen-P) was extracted with a solution of $NaHCO_3$ and quantified by reaction with ascorbic acid, following the standard method AFNOR NF ISO 11263. Soil nickel availability was evaluated after extraction with DTPA-TEA (0.005 M DTPA with 0.01 M $CaCl_2$ and 0.1 M triethanolamine (TEA) at pH 7.3, 1:2 w/v, 2h shaking) (Lindsay and Norvell 1978). Soil subsamples were ground in a ceramic mortar and 0.5 g of dry ground soil were digested in 2 mL of concentrated HNO_3 and 6 mL of concentrated HCl on a hot plate at 105°C. The final solutions were filtered (0.45 μm DigiFILTER, SCP science, Canada) and brought to volume (50 mL) with deionised water. Pseudo-total soil concentrations of Ca, Co, Cr, Fe, K, Mg, Mn, Ni, P and S were determined by analysis of the digests with ICP-AES (Liberty II, Varian).

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

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232 Green branchlets were cleaned with tap water and rinsed with deionised water. Litter samples and

233 cleaned branchlets were dried (60ºC) 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 HNO_3 and 5 mL of H_2O_2 (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' = -Σ 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 Mg^{2+} in

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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

9

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

10

NMDS analyses revealed differences in plant community composition related to *C. terminale* litter cover at both sites. In Wuluh site (2D stress = 0.18) plant communities in sampling points were grouped according to tree age classes. Major differences were found among age-classes and only small overlaps occurred between plant communities around S and YT, whereas communities around mature trees were clearly separated (Fig. 7A). This is congruent with a higher cover of several shrub and tree species (e.g. *Macaranga kinabaluensis* –MAKI–, Euphorbiaceae; *Rapanea sp.* –RASP–, Myrsinaceae) as well as pitcher plants (*Nepenthes sp.* –NESP–, Nepenthaceae) that were absent 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) and MT (H' = 1.56, p = 0.003), whereas no difference was found between YT and MT.

In the case of Kulung, the space occupied in NMDS plot by plant communities growing on litter layers around MT greatly overlapped the space occupied by communities from NV Technosol (i.e. low differences in species composition and cover) (Fig. 7B). The most important differences could 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 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) ($F_{1,14}$ = 0.577, p = 0.460).

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 non-vegetated 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 morpho-physiological 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.

It is known that inoculation with *Frankia* improves the nutrient status of the actinorhizal plant and enhances its growth (Sayed 2011). However, the abundance of *Frankia* in soils and its nodulation

11

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

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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 N_2 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) (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.
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

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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

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communities was observed around mature trees growing on soil disturbed by landslides. Overall, *C. terminale* appears to be a promising species for the restoration of degraded serpentinite soils in SE Asia and Melanesia. However, the application of this species in ecological restoration programs need the development of more research in: (i) plant reproduction, including knowledge about seed viability and strategies for vegetative multiplication; (ii) agronomical practices, especially in relation to planting density and management of litter layers to favor establishment of new species, and, (iii) exploration of soil biota, with special emphasis on symbiotic arbuscular mycorrhiza fungi and also saprophytic fungi able to degrade *C. terminale* litter.

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497

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_{H_2O}	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

20

504
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)			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

21

512 **Table 3** Chemical composition of *C. terminale* photosynthetic branchlets and litter. Mean values for
513 branchlets (n = 15) and litter (n = 9 - 15) are presented for each site and averaged for all samples.
514 Units are indicated for each variable. P and S concentrations in litter are below limit of
515 quantification (P, 125 mg kg^{-1}; S, 525 mg kg^{-1}), but are presented for comparative purposes.
516

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

517
518 **Table 4** Two-way ANOVAs of N, P, K and Ca concentration in branchlets of *C. terminale* of two
519 ultramafic sites (Kulung and Wuluh) and 3 age-classes (sapling, young tree and mature tree; for
520 each site and age-class, n = 5). Site and age-class were fixed factors. Each line corresponds to one
521 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

22

FIGURE CAPTIONS

523

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

530

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

537

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
541 each of the samples (for each site, n = 9-10 in NV class and n=5 in S, YT and MT classes). For each
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; *CaMg*, 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

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

554

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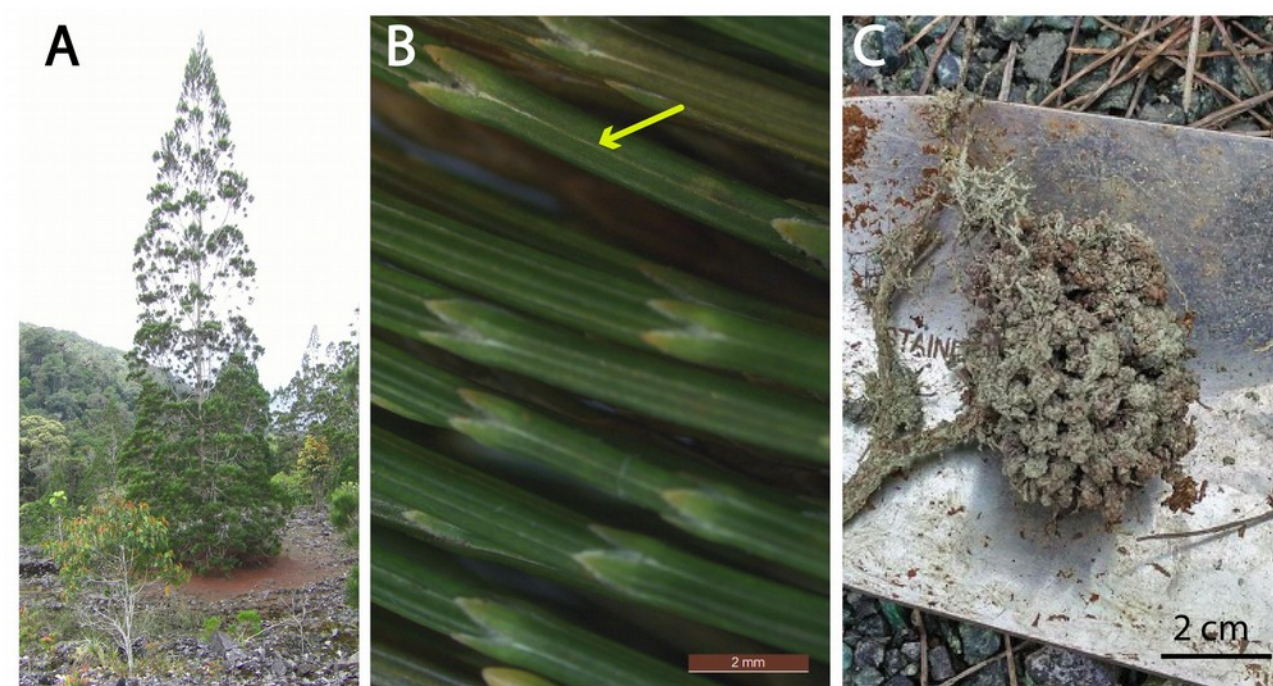560 thick mineral element to the right.

561

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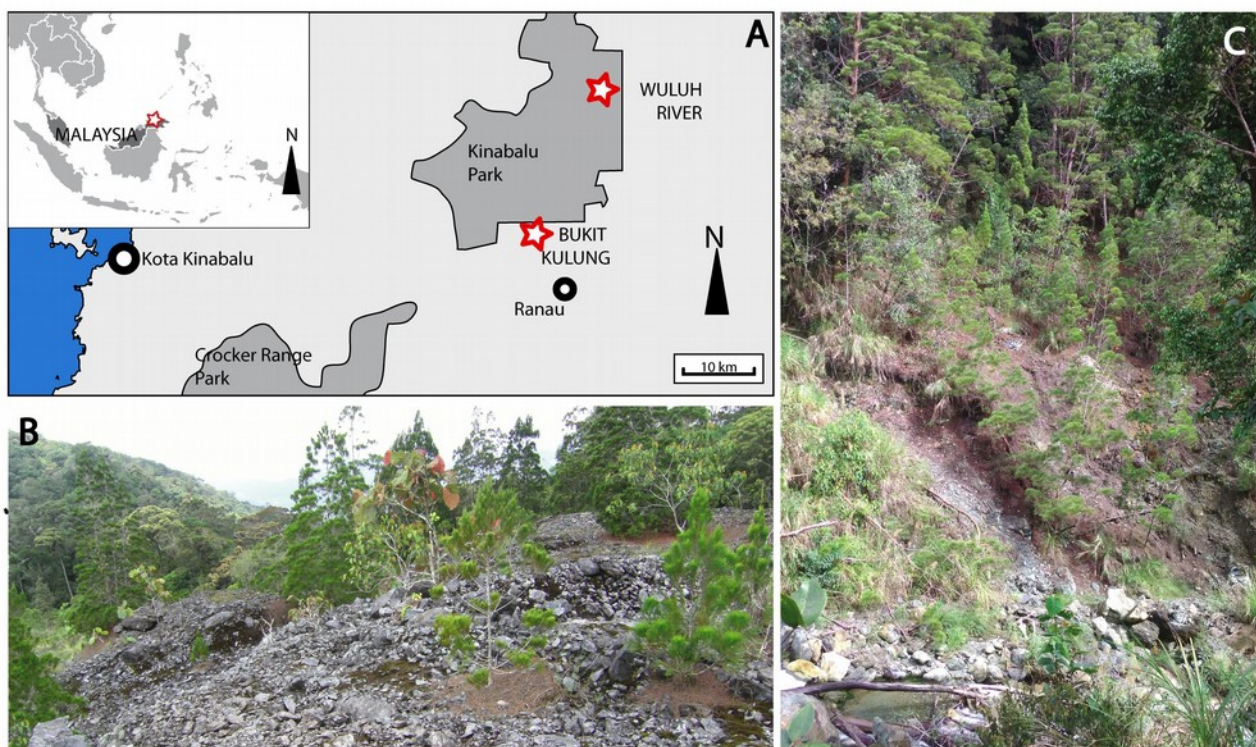566 Tukey post-hoc test) among age-classes in Kulung and Wuluh, respectively.

567

568 **Figure 7** NMDS analyses of plant communities around *C. terminale* plants at Wuluh (**A**) and
569 Kulung (**B**) sites. Colored polygons are convex hulls connecting plant communities on bare soil
570 (gray), and around *C. terminale* saplings (blue), young trees (yellow) and mature trees (green) (for
571 each class, n = 8 at Kulung and n = 5 at Wuluh). Red letter codes indicate plant species (see
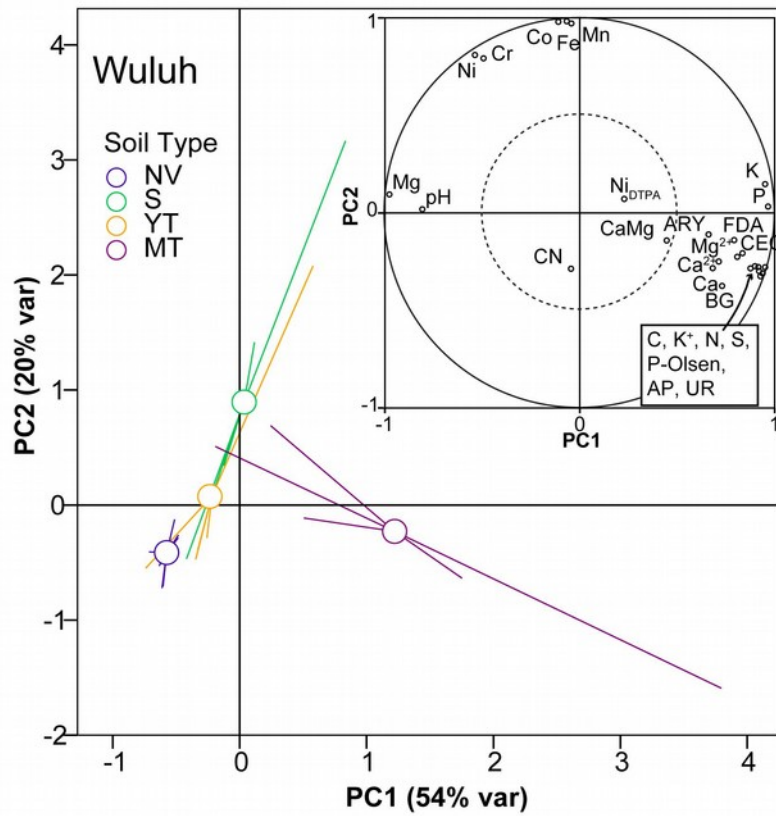572 supplementary material Table S2).
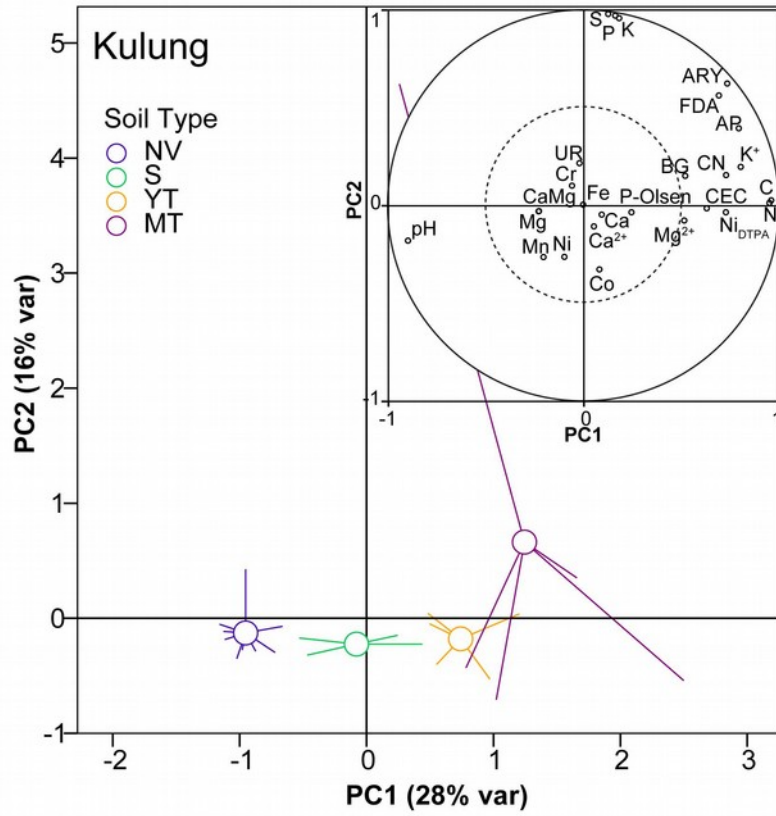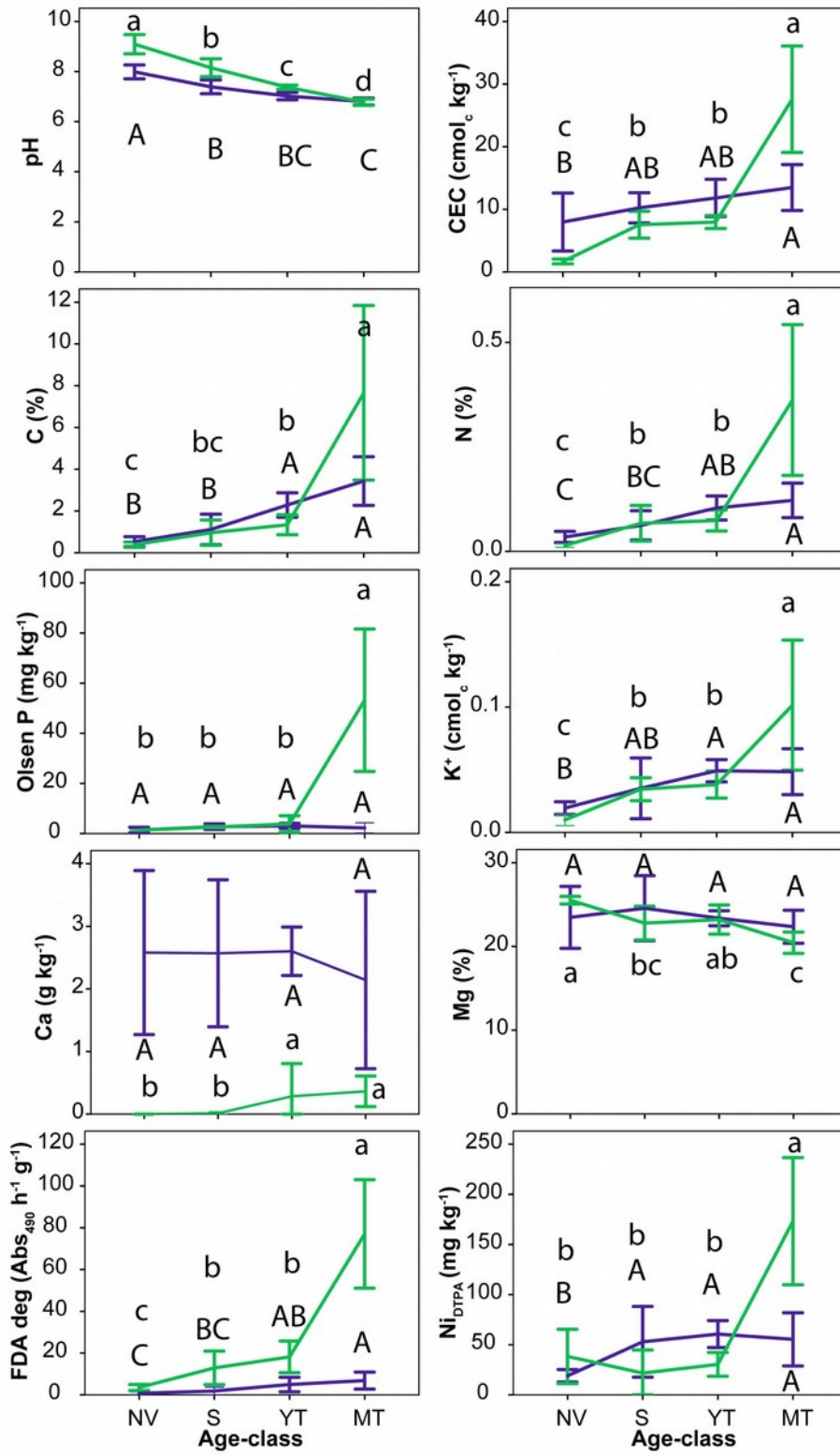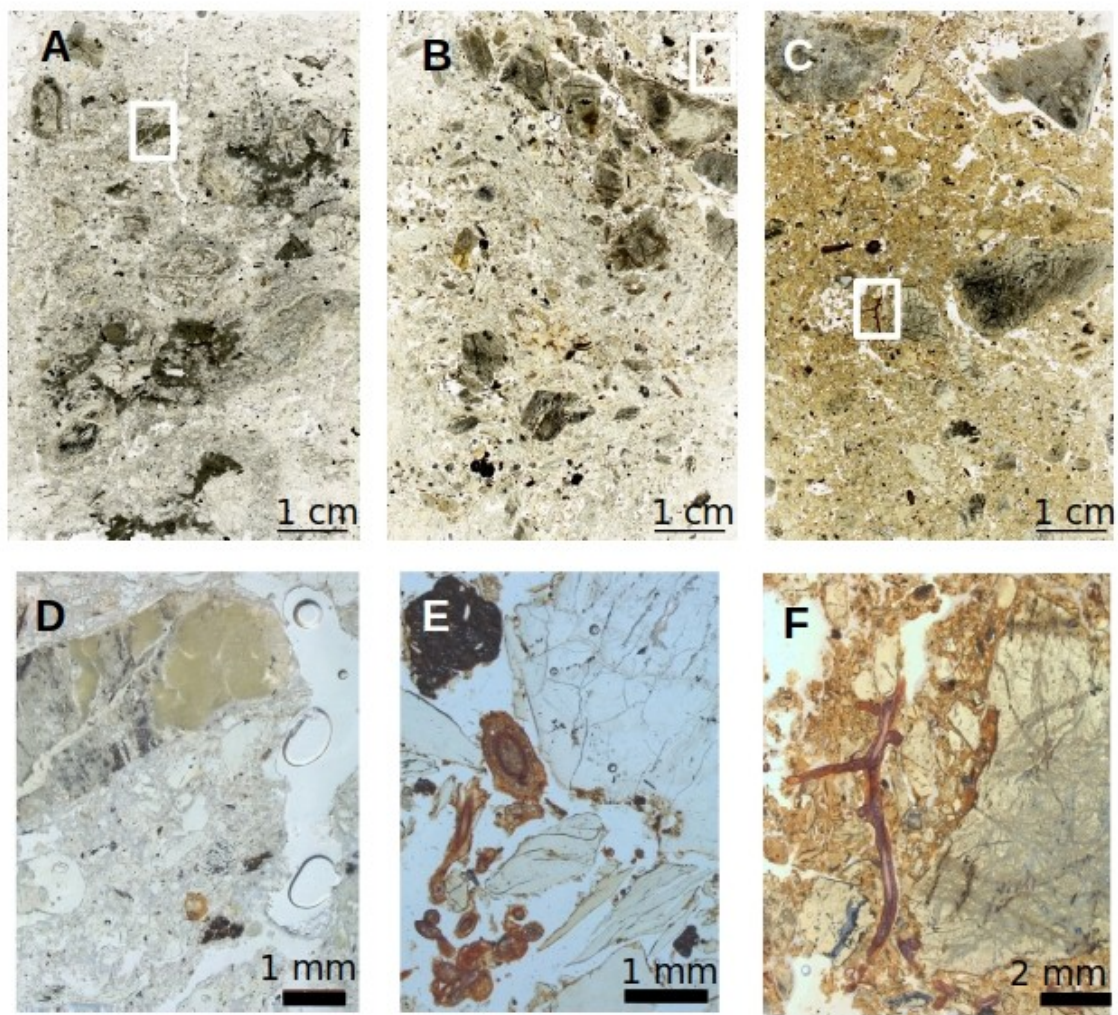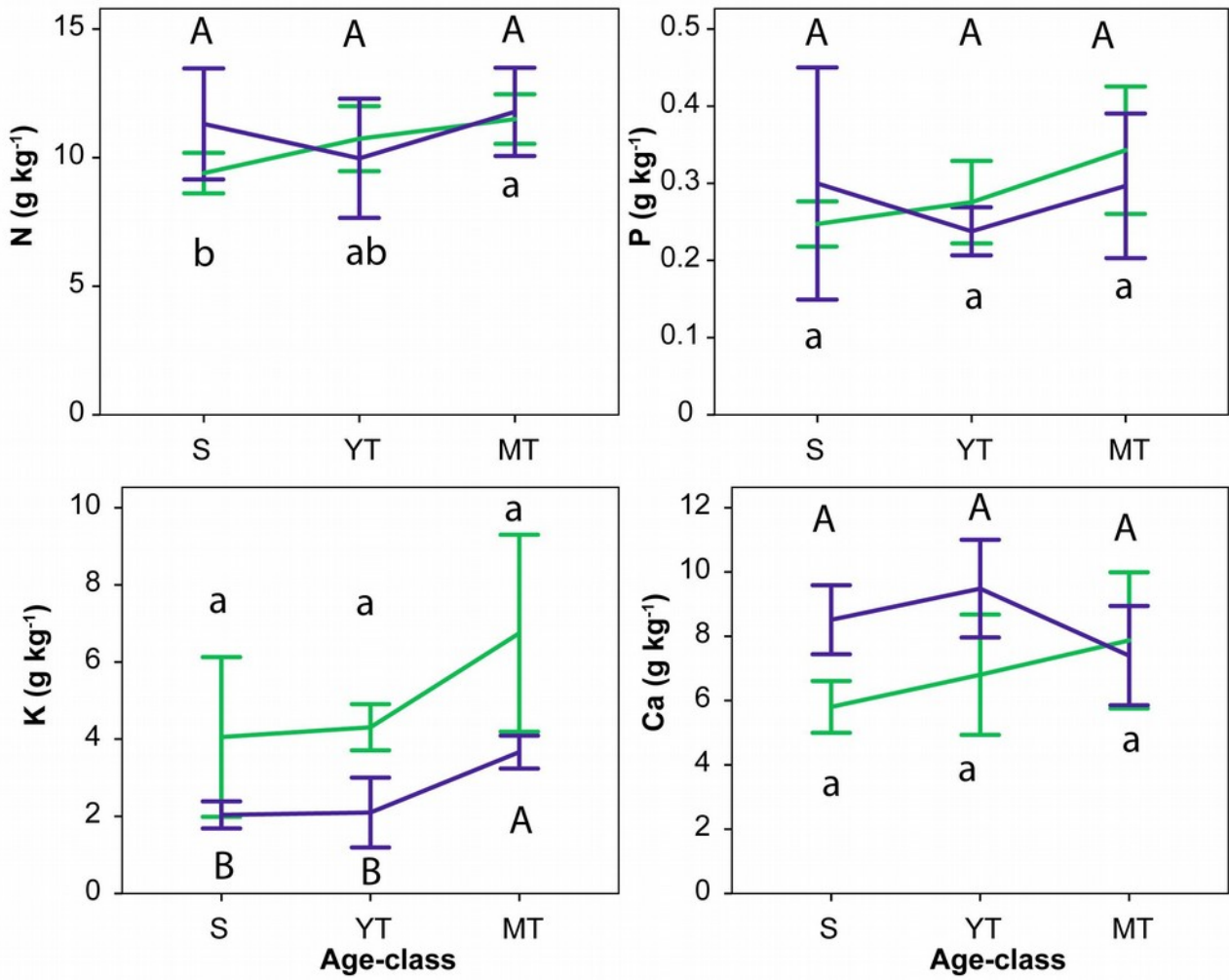
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23

574   Fig. 1:

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575   Fig. 2:

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578   Fig 3:
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Fig. 6: