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1 **Contrasting effects of long term phosphorus fertilization on glomalin-** 2 **related soil protein (GRSP)**

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8 **Abstract**

9 Glomalin-related soil protein (GRSP) is believed to be produced by arbuscular mycorrhizal
10 fungi (AMF). However, this fraction of organic matter is influenced by various soil and land-
11 use parameters and its link with AMF has been questioned. The aim of this study was to advance
12 the understanding of the origin of GRSP and its value as a marker of AMF activity by focusing
13 on the effects of soil P status. Archived soils from two phosphorus fertilisation field trials on
14 sandy soils under maize cultivation in the south-west of France were studied. Trends in GRSP
15 and soil organic carbon (SOC) were compared. Grain yield and available P (Olsen-P) were
16 monitored and compared to assess P sufficiency/limitation. The time trends of GRSP for each
17 site were not significant. No significant P-fertilization effect on GRSP was observed for the P-
18 sufficient continuously cropped soil, for which the crop yield increase was small. For the P-
19 deficient, former forest soil, P fertilization led to a marked increase in crop production and a
20 significantly larger GRSP content. These trends are coherent with GRSP input linked to crop
21 C-inputs, including the incorporation of crop residue.

22 **Keywords:** glomalin; Autoclaved-citrate extractable (ACE) protein; phosphate; fungal activity;
23 long-term field trial

24 Glomalin-related soil protein (GRSP) is an empirically defined component of soil organic
25 matter that has attracted much attention since it was first reported [1]. It is now recognized to
26 be a complex mixture of proteins and non-proteins [2]. It was initially claimed to be of
27 arbuscular mycorrhizal fungal (AMF) origin, and has been recommended as a marker of fungal
28 activity [3, 4], however this is increasingly challenged [5-7]. Holátko et al questioned whether
29 correlations between GRSP and AMF indices are sufficient to validate GRSP as an AMF proxy
30 [5]. Although GRSP content is often found to increase with mycorrhizal inoculation, there is
31 often poor correlation between GRSP content and other markers of fungal activity [5, 8-11].
32 Despite the importance of mycorrhizal infection for P nutrition, there have been few studies of
33 the relation between GRSP and soil P-status. Available phosphorus (P) is known to have
34 contrasting effects on AM fungal abundance in soil. Mycorrhizal infection and activity increase
35 following P-fertilization of severely P-limited soil, but decrease when P is in excess [12-14].
36 Comparisons of soil-P and GRSP include studies of organic versus mineral fertilizers or
37 statistical comparisons of GRSP, other AMF markers and edaphic properties of contrasting soils
38 [4, 15-18]. A short-term pot experiment using acid-washed sand found different effects of P on
39 GRSP and AMF [19], whereas strong effects of available P have been reported on fungal
40 composition [10].

41 The aim of this study was to investigate the effect of long-term phosphorus fertilization on the
42 GRSP fraction in order to contribute to the elucidation of the origin of GRSP. We chose to
43 investigate archived soils from two long-term field trials of phosphorus fertilization. This
44 allows the time trend to be established, but precludes direct measurements of other markers of
45 fungal composition and activity, since they must be carried out using fresh soil.

46 Both field trials were established on sandy soils under irrigated maize crop production in the
47 region of Bordeaux, South-West of France and arranged in randomized block designs with four
48 replications. The crop residues were crushed and ploughed into soil (25 cm depth) before

49 sowing. More information on the sites and trials are reported elsewhere [20, 21]. Fertilisation
50 (including N and K to be non limiting) was applied in spring. One site, named Pierroton, was
51 converted from forest two years before the start of the trial. For the other site, named Tartas,
52 the soil was under continuous mixed cultivation prior to the trial. Site descriptions and
53 treatments are summarized in Table 1. Soils were sampled in the surface layer (0-25 cm)
54 roughly every four years before fertilisation, air-dried, sieved < 2 mm and stored until required.
55 Archived soil from duplicate plots of two treatments were selected from each trial for the
56 present study; zero or very low P fertilisation (P_0) or excess P-fertilisation (+P). Soil was further
57 crushed and sieved < 200 μm prior to GRSP extraction and quantification to ensure
58 homogeneous sampling. The C-content of this clay+silt sized fraction was about twice that of
59 the whole (<2 mm) fraction. GRSP was extracted in triplicate according to the usual procedure
60 (1:8 soil:solution g ml^{-1} ratio in neutral 20 mM sodium citrate solution autoclaved for 30 min
61 at 121°C). Protein in each extract was quantified in triplicate using the nonspecific colorimetric
62 Bradford method after recommended dilution and colour correction of the absorbance at 595
63 nm [22, 23]. C and N contents were quantified on the <200 μm soil samples by elemental
64 analysis.

65 Analyze of variance (ANOVA) were used to investigate changes in GRSP and SOC depending
66 on treatment and trial period. These analyses were carried out using the free software
67 environment R [24].

68 Figure 1 shows for both trials, the average of data of two plots for each P-fertiliser treatment.
69 In the cleared forest site (Pierroton), grain production declined rapidly in the absence of P-
70 fertiliser but was immediately restored and maintained at about the initial production when
71 limited P was supplied after 7 yr. Excess P-fertilisation (+P) gave an initial increase in grain
72 production which then levelled off. Overall the effect of P-treatment on grain yield was highly
73 significant ($P < 0.001$), but neither trial period nor the interaction between treatment and trial

74 period were significant (Table SM_1). Available P increased continuously in the +P soils and
75 decreased in the absence of P-fertilization then recovered and stabilised when sub-optimal P
76 was applied. P-treatment, trial period and their interaction also had highly significant effects on
77 soil-P availability ($p < 0.001$, Table SM_1). In the Tartas, previously farmed site, grain
78 production increased significantly ($P < 0.001$) and continuously for both P_0 and +P soils, with a
79 small significant yield increase (+9%, $P < 0.001$) with the +P treatment. Available P was
80 correspondingly greater in the +P treatment ($P < 0.001$) but with no time effect. C content was
81 initially greater in the cleared forest site than the farmed site, with no evidence of C depletion
82 following the introduction of maize cropping, in contrast to the rapid decrease often observed
83 following deforestation in temperate zones [16–18]. The conservation of SOC may be due to
84 the mulching with crop residue. At Tartas, there was a small, slightly significant decrease of
85 SOC with time ($P < 0.05$), but no significant P-fertilisation effect was observed.

86 GRSP content was greater at the previously forested Pierroton site than for the continuously
87 farmed Tartas site, in accordance with the previously reported land-use effect of GRSP content
88 [7]. GRSP content for the Pierroton site showed considerable inter-plot variability, especially
89 in the early stages of the trial. ANOVA showed that both GRSP and GRSP/SOC were
90 significantly lower in the P_0 than the +P plots ($P < 0.01$). This points to GRSP input linked to C-
91 dynamics related to crop production and residue application, rather than to fungal activity.
92 Straw addition has been reported to enhance GRSP [25, 26]. For Tartas no coherent time trends
93 or P-effects were apparent on C content, and there was no significant treatment or time effect
94 on GRSP or GRSP/SOC. This suggests that SOC and GRSP may already have been at steady
95 state for agricultural land-use at this site. The accumulation of available P prior to the trial may
96 have inhibited fungal activity, and further, excess additions of P would therefore have had little
97 or no impact on either primary production or fungal activity. According to the ANOVA
98 analysis, there was no significant change in GRSP content at either site during the time of the

99 trials (>0.05). This suggests a strong legacy effect with a relatively small shift in GRSP content
100 in response to land management changes. The absence of a strong time effect on GRSP indicates
101 that GRSP content results from a dynamic balance between input and turn-over.

102 In conclusion, GRSP content did not change markedly during the long-term (20-30 year) trials.
103 When P fertilization was in excess with little effect on crop yield, no effect was observed on
104 GRSP. In P-deficient soil, P fertilization increased both crop yield and GRSP content.
105 Accounting for this trend assuming GRSP to be a product of fungal activity, would have the
106 unlikely implication that P addition to a P-deficient former forest soil increased fungal activity.
107 The greater GRSP content and its enrichment within organic matter following P-addition to P-
108 deficient soil are coherent with GRSP being directly or indirectly linked to crop production.
109 Direct links would arise from greater root activity during growth and changes in fungal
110 composition and activity, and indirect effects would include the stimulation of GRSP content
111 by greater residue incorporation, as previously observed for straw. The rather small change in
112 GRSP following land-use change (forest to cropland) suggests that GRSP is a stable fraction of
113 SOC.

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117 **Figure and Table Captions**

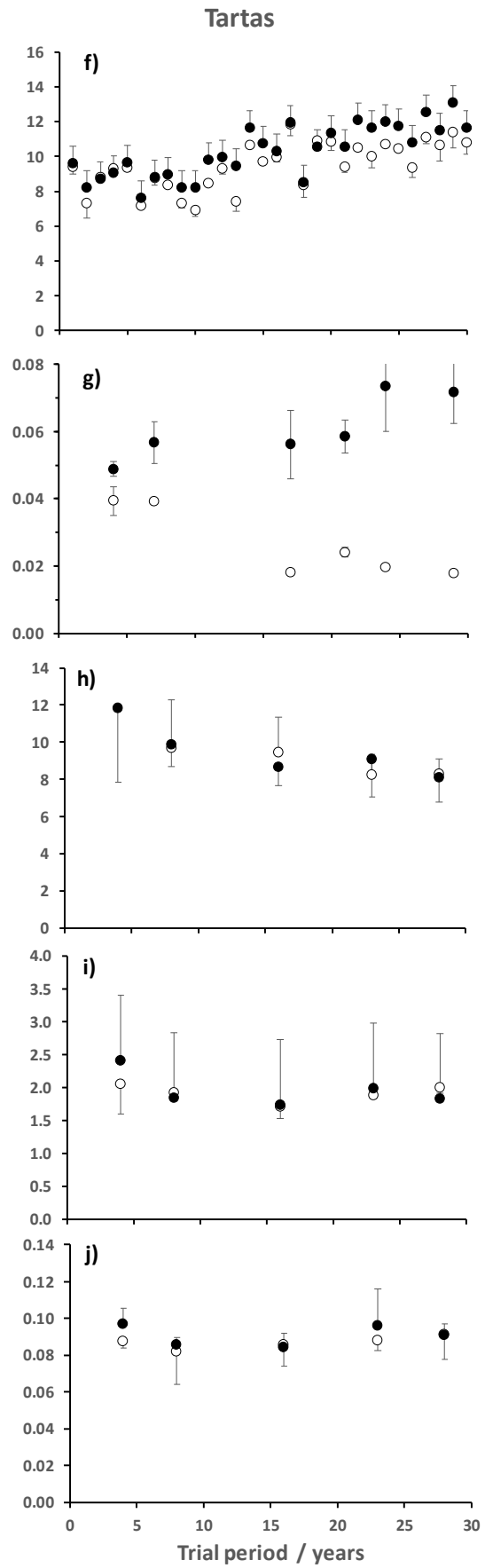
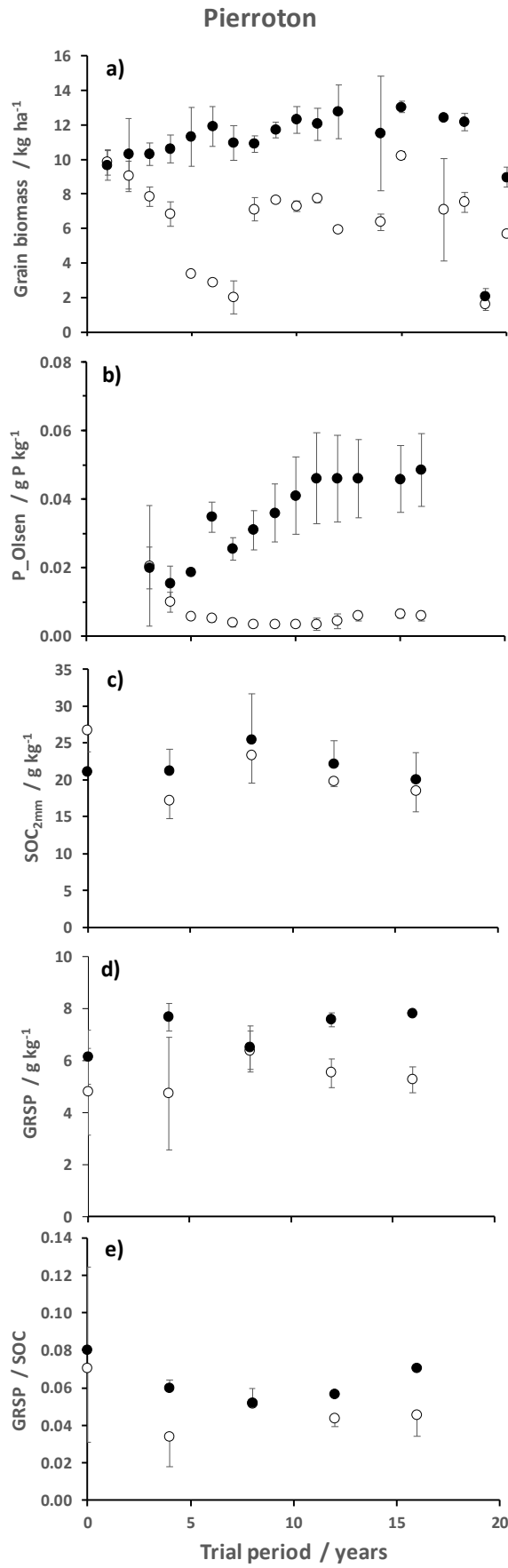
118 **Figure 1.**

119 Grain dry mass production (a, f), Olsen P content of air-dried sieved soil (<2 mm) (b, g), SOC
120 of air-dried sieved soil (<2 mm) (c, h), GRSP content of < 200 μm size fraction (d, i) and GRSP
121 relative to C-content of the <200 μm size fraction (e, j) for the Pierroton deforested site (a-e)
122 and Tartas continuously farmed site (f-j). Open Symbols indicate control (**P₀**) and closed
123 symbols phosphorus addition (**+P**). Bars (positive or negative for clarity) indicate standard
124 deviation between plots. Analytical standard deviation between triplicates was about 5% and
125 standard deviation between triplicate subsamples of soils about 7%.

126 **Table 1.**

127 Site location, soil class and summary of P treatments for plots chosen for the study.

128 **Figure 1**



129

130

131 **Table 1.**

Site name	Pierroton	Tartas
Coordinates	44° 44'N, 0° 46' W	43°52'N, 0°44W
Altitude / m	60	55
Mean annual temperature / °C	13.5	13.6
Mean annual rainfall / mm	950	917
Period of experiment (duration / year)	1995-2015 (20)	1972-2000 (28)
Previous crop	Pinus forest	Mixed farming, mostly maize
Soil Class : World Reference Base for Soil Ressources	Podzol	Arenosol
*P fertilisation P₀	6.7 (0, then 10 after 7 years)	0
*P fertilisation +P / kg P ha ⁻¹ yr ⁻³	91 (120, then 80 after 7 years)	96

132 *For the Pierroton site P additions varied over the Trial period, the average P-addition for each
133 treatment is given and in brackets the range of additions. More information on the full trial
134 treatments may be found in [21] for the Pierroton site and [20] for the Tartas site.

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