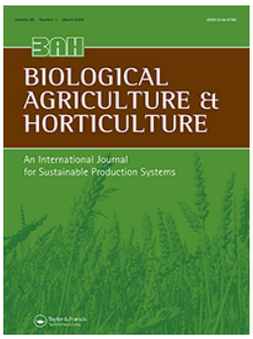

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Growth responses of garden cress (*Lepidium sativum* L.) to biodynamic cow manure preparation in a bioassay

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ABSTRACT

Natural substances are extensively used as biostimulants in agriculture. Notably, horn-manure preparation (HMP) is fermented cow manure sprayed at low concentrations onto biodynamically cultivated fields. The present study investigated the effect of HMP on the growth of garden cress (*Lepidium sativum* L.) cultivated in a bioassay (randomized block design, $n = 20$). Seedlings were cultivated in a water medium. Treatments of a drop of HMP suspension (1 µl or 0.1 µl) or of water (Control) were added to the medium. Long-term series of trials, with two different HMPs, were conducted over 18 and 9 months with 76 and 38 trials, respectively. In the first series, the effect of a 1 µl drop of HMP suspension on root growth was significant overall (−2.4%, $p = 0.004$, Tukey-Kramer-test) and in 35.5% of the individual trials ($p < 0.05$). However, the effects fluctuated strongly between the trials (from −25.7% to +19.1%). The effect of a 0.1 µl drop was similar, but lower in magnitude. The results of the second series were analogous. Comparison of statistical models provided significant evidence of a growth-stabilising effect. An additional series of 22 negative control trials indicated an acceptable false positive rate. It was concluded that HMP, at low doses, significantly influenced root growth at early stages, with a stabilising pattern of action. Further development of the bioassay should improve its power and stability over time. A stabilising effect may induce an increased resilience of the agricultural system.

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Introduction

Development of agricultural practices currently tends to focus more and more on sustainability and product quality instead of steady productivity and increased yield. The new challenges require innovative methods like the use of biostimulants that are applied at low doses to activate physiological processes (Sharma et al. 2014; Brown and Saa 2015; Bulgari et al. 2015; Nardi et al. 2016; Yakhin et al. 2017). They have the potential to stimulate plant development, enhance crop quality or reduce stress effects (Calvo et al. 2014; Bulgari et al. 2015; Du Jardin 2015).

The use of biostimulants is a characteristic practice in biodynamic (BD) agriculture with eight preparations from mineral, plant or animal-derived ingredients (Koepf et al. 1979). These BD preparations are applied with farm manure or sprayed on the fields, typically in small quantities. In the European Union, the use of BD preparations for organic production is authorised through the EU-regulation 834/2007 (Council of the European Union 2007).

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Supplemental data for this article can be accessed here.

The influence of the application of BD preparations on crop quantity and quality as well as on soil characteristics has been indicated in some studies (e.g. Carpenter-Boggs et al. 2000; Zaller and Köpke 2004; Jariene et al. 2015), but has also been contested (Berner et al. 2008; Döring et al. 2015). In their review articles, Turinek et al. (2009) and Geier et al. (2016) noted that BD preparations showed effects on yield, soil quality and biodiversity. By contrast, Chalker-Scott (2013) highlighted that many studies did not show significant results and that convincing data supporting the efficacy of biodynamic preparations were scarce.

The shortage of clear and conclusive data can be partly explained by a methodological bias. Indeed, the studies conducted so far overwhelmingly consisted of practice-oriented field trials with limited control of environmental factors, resulting in fluctuating trial conditions. In contrast to this field approach, laboratory tests have rarely been employed, although bioassays are a major instrument in plant physiology (Audus 1972), ecotoxicology (OECD 2009) and medicine (Agarwal et al. 2014; Butterweck and Nahrstedt 2012; Jäger et al. 2015) to investigate the bioactivity of low-dosed substances. In agriculture, bioassays have also been extensively used to investigate the hormone-like bioactivity of biostimulants (Ertani et al. 2011; Colla et al. 2014).

The present study shows the first results from the development phase of a specific bioassay to test the bioactivity of one BD preparation, the horn-manure preparation (HMP). HMP consists of a humus mixture obtained from fermented cow manure. In BD practice, a HMP-water suspension with a concentration of 3 g HMP l^{-1} is applied onto the fields at 40–100 l ha^{-1}. According to BD fundamental and practical principles, the working hypothesis is that HMP affects root development during the early germination process. This assumption guided the choice for a bioassay developed by Baumgartner et al. (2014) using cress seedlings (*Lepidium sativum* L.) as a test organism, providing a simple way of testing early root development with high reproducibility and accuracy.

The goals of the present study were to determine (1) the sensitivity of the growth of the cress to low-doses of HMP, (2) the stability over time of this bioactivity, (3) its pattern of action, and (4) its dose-response relationship. These goals corresponded with the suggestion of Yakhin et al. (2017) to focus the research on biostimulants on efficacy and on determining a broad pattern of action.

Materials and methods

Materials: HMP suspension and cress seeds

The HMPs and the HMP water suspension used in this investigation were produced according to the biodynamic criteria (Koepf et al. 1979). The HMPs were produced in 2010 and 2012 at the research site at Landbauschule Dottenfelderhof, Bad Vilbel, Germany. Manure from several cows was collected and placed in cow horns, which were then buried in the soil during the winter and unearthed in spring. The HMP was the 'humus mixture' that resulted from this fermentation.

A new HMP water suspension was produced for the setting up of each trial. It consisted of 21 g of HMP in 7 l water collected from a drilled well at the research site. Basic analysis of the well-water (Krohm Wassertechnik GmbH, Karlstein, Germany) was performed on two occasions over the experimental period (Table 1).

Table 1. Basic analysis of the well-water.

	Analysis 1	Analysis 2
Date	11 January 2012	6 December 2012
pH	9.01	8.60
Conductivity (µS cm^{-1})	1625	1588
HCO_3^- (mg l^{-1})	378.4	422.4
Cl^- (mg l^{-1})	171.6	144.7

Organically certified cress seeds were obtained from Bingenheimer Saatgut AG (Echzell, Germany). Seeds that were damaged or deviated in size, shape or colour were removed.

Experimental procedure

The experimental procedure was a variation of the procedure described by Baumgartner et al. (2014) and consisted of the hydroponic cultivation of cress seedlings in hanging bags. LD-PE bags (Minigrip ® 120 × 170 mm, Inteplast Group, USA) were filled with 6 ml of drilled well-water, collected from the research facility, as cultivation medium. Chromatography paper (FN 1, Sartorius AG, Germany) was introduced into each bag. 16 cress seeds were aligned on the soaked chromatography paper 10 cm above the bottom of the bag (Figure 1). The treatments consisted of the application of a drop of the suspension onto the chromatography paper with a microliter syringe (Acura 825, Socorex Isba S.A., Switzerland) 5 to 7 hours after seed imbibition. The drop was applied in the middle of the bag, about 2 cm above the seeds. It consisted of either (i) 1 µl well-water (Control), (ii) 0.1 µl HMP suspension ($D_{0.1µl}$) or (iii) 1 µl HMP suspension ($D_{1µl}$). Each trial consisted of 60 bags, 20 for each of the three treatments. The bags were suspended from hangers placed in a light-isolated incubator (KB 720, Binder GmbH, Germany) at 19°C according to a one-factorial randomised complete block design.

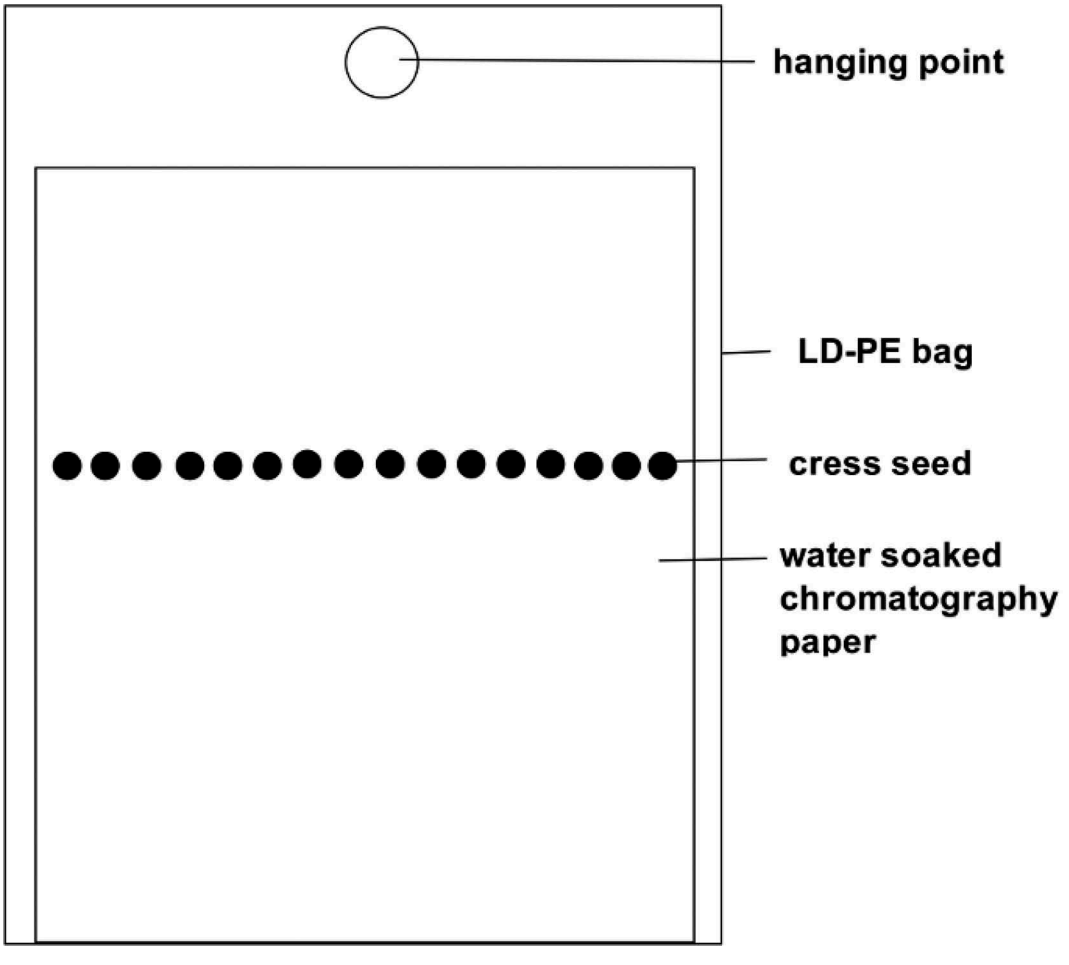

Figure 1. Schematic layout of one bag.

Root and hypocotyl growth were marked daily after the second day with a point on the bags, 1–2 mm aside of the position of the root and hypocotyl tips. During this operation, the bags were taken from the hangers in the incubator and placed on a table at room temperature for 30 ± 10 min. Trials were stopped as early as 7 days after the start. The bags were then photographed and the daily growth of roots (from day 2–7) and hypocotyl (from day 3–6) was assessed according to the points marked on the bags via image analysis software (Sigma Scan Pro 5.0, SPSS Inc., USA).

Treatments were not blinded during the drop application phase, because this required the addition of two drop volumes (0.1 µl and 1 µl) that were necessarily different. In all other steps, treatments were blinded by using coded bags. Sample decoding took place at the very end of the experiment, after all length measurements were accomplished. Exclusion of experimental material was blinded as well and occurred during the marking operation, as seedlings with skewed or retarded growth were visually identified and not considered. Data of bags with fewer than 10 recorded seedlings were discarded.

The choice of well-water as cultivation medium was based on experiments in which standardised solutions (distilled water or nutrient solutions) were investigated. The unpublished results indicated that HMP effects were detected mainly in the well-water. Moreover, the well-water was representative of natural variability, which was considered to be advantageous for determining a broad pattern of action. However, the well-water fluctuated in quality, and the search of a standardised cultivation medium was postponed for further development (article in preparation).

The application of the HMP suspension as one drop at the early stage of seed imbibition mimicked BD practice (dispersion in droplets on the fields at sowing). But the resulting dispersion of the HMP suspension was non-uniform. Hence, the HMP concentration in the bag solution can only be roughly estimated as 0.05 mg l^{-1} for treatment $D_{0.1µl}$ and 0.5 mg l^{-1} for treatment $D_{1µl}$. These concentrations corresponded to the estimated HMP concentration in the soil water in the BD practice at 0.4 mg l^{-1} (Giannattasio et al. 2013).

Performed trial series

The treatment factor was investigated over long-term trial Series A (76 independent trials, 18 months) and B (38 trials, 9 months) from July 2011 until February 2013 (Table 2, Figure 2). In Series A and B, treatments were Control, $D_{0.1µl}$ and $D_{1µl}$. The HMP that was investigated in Series A was produced in 2010 and that for Series B was produced in 2012. Series C (22 trials) was a negative control trial series in which all three treatments were Control (pseudo-treatments). In this way, the rate of false positive results (significant differences between the Control treatments) was investigated to determine the reliability of the bioassay. The trials within a series were performed successively on a weekly basis.

Statistics

A total of 136 trials with 130,560 seeds in 8,160 bags were conducted. This experimental data was analysed with different statistical approaches.

The following linear mixed model was applied for the analysis of each individual trial:

$$Y_{ijk} = \mu + b_i + w_j + bw_{ij} + e_{ijk} \quad (1)$$

Table 2. Overview of the trial series A, B and C.

Series	A	B	C
Time period	6 July 2011–14 January 2013	27 April 2012–9 February 2013	21 May 2012–15 October 2012
Number of trials	76	38	22
Treatments	C, $D_{0.1µl}$, and $D_{1µl}$	C, $D_{0.1µl}$, and $D_{1µl}$	C_1, C_2 and C_3
HMP production year	2010	2012	-

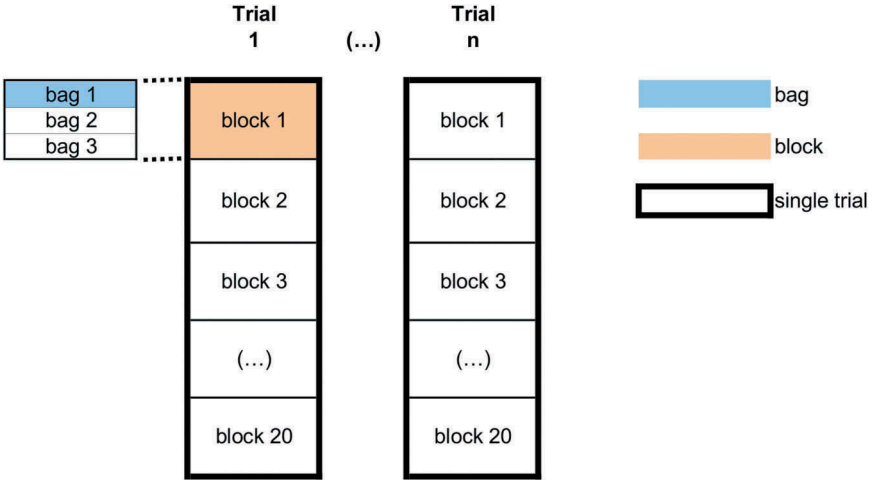

Figure 2. Schematic layout of the series design. The randomisation unit was the bag (in blue), in which a drop of liquid (water or HMP suspension) was applied. A block unit (in orange) consisted of three bags. A trial consisted of a randomised complete block design with 20 blocks. The series A, B and C comprised 76, 38, and 22 (= *n*) trials, respectively.

with μ as the overall effect, b_i the fixed effect of the i-th treatment (i = 1–3), w_j the random effect of the j-th block (j = 1–20), bw_{ij} the random effect of the ij-th bag, e_{ijk} the random effect of the k-th seedling (k = 1–16) in the ij-th bag, and Y_{ijk} the root or hypocotyl length of the k-th seedling in the ij-th bag.

For the whole series, meta-analyses were conducted by considering the mean length measured for each bag (Madden et al. 2016). If the sample size is nearly the same between the bags, the means still allow for an approximately valid analysis (Piepho 1997a). Data from 4,499, 2,253 and 1,312 bags were analysed with the following model for Series A, B and C, respectively:

$$Y_{ijk} = \mu + b_i + t_j + bt_{ij} + w_{jk} + e_{ijk} \quad (2)$$

with μ the overall effect, b_i the fixed effect of the i-th treatment (i = 1–3), t_j the random effect of the j-th trial (Series A: j = 1–76; B: j = 1–38; C: j = 1–22), bt_{ij} the random effect of the interaction between the i-th treatment and the j-th trial, w_{jk} the random effect of the k-th block in the j-th trial (k = 1–20), e_{ijk} the error effect of the ijk-th bag, and Y_{ijk} the mean root or hypocotyl length in the ijk-th bag.

Inspection of the data suggested that the HMP treatment had a stabilising effect, meaning that in trials with above-average growth the HMP-treated seeds tended to grow less than the Control, whereas in trials with below-average growth HMP-treated seeds grew better than the Control. This was detected by plotting HMP means versus Control means. However, inference for this regression was not straightforward because both variables were subject to estimation error (Fuller 1987). For this reason, a Finlay-Wilkinson regression (Finlay and Wilkinson 1963) was considered to compare treatment means per trial against trial means. This regression suffered from the same errors-in-variables problem, but this could be addressed by the following model:

$$Y_{ijk} = \mu + b_i + c_i * t_j + d_{ij} + w_{jk} + e_{ijk} \quad (3)$$

where c_i is the slope of the regression of the i-th treatment on the random trial effect t_j and d_{ij} the deviation from the regression. This regression obeys the constraint that the mean of the three regression slopes c_i (i = 1, 2, 3) equals one.

It was noted here that Model (3) implied a factor-analytic variance-covariance structure, which facilitated fitting this model using residual maximum likelihood (Piepho 1997b) and effectively accounted for the error-in-variables problem (Fuller 1987) of classical Finlay-Wilkinson regression.

Furthermore, the heterogeneity of variance was considered. Model (2) was fitted by considering variance homogeneity (hom) or heterogeneity (het) for the random effect bt_{ij}. In the following, the resulting models were called $(2)^{hom}$ and $(2)^{het}$, respectively. In the same way, Model (3) was fitted by considering variance homogeneity (Finlay and Wilkinson 1963) and treatment-dependent variance regarding the random deviation d_{ij} (Eberhart and Russell 1966). These model variations were called $(3)^{hom}$ and $(3)^{het}$.

The hypothesis of a stabilising effect corresponds to an interaction variance that is smaller for HMP treatment than for the Control. Furthermore, the hypothesis of a stabilising effect *depending on experimental conditions* (a regulating effect) corresponds to a slope c_i in Model (3) that is smaller for HMP treatment than for the Control.

The four models were compared with residual likelihood ratio tests (Verbeke and Molenberghs 2000). If the models differed significantly, the final meta-analysis was conducted using the model found to have the best fit. If not, the final meta-analysis was conducted with the simplest model. Furthermore, the Akaike Information Criterion (AIC) was computed for all four models, with smaller values indicating better-fitting models (Verbeke and Molenberghs 2000).

The analyses for Series A, B, and C and for the comparison between Models (2) and (3) are presented in the results sections below. Under the null hypothesis $H_{0,}$ the two models do not differ, meaning that all three treatments respond equally to changing experimental conditions (H_0: $c_1 = c_2 = c_3 = 1$). Alternatively, they respond differentially and may be suggesting a growth-stabilising effect.

The influence of the non-uniform dispersion of the HMP suspension in the bags (due to the drop application) was investigated as well. A meta-analysis with the data of the seedlings was performed with the following model derived from Model (2):

$$Y_{ijkl} = \mu + b_i + p_l + pb_{il} + t_j + bt_{ij} + w_{jk} + e_{ijkl} \quad (4)$$

with p_l the fixed effect of the lth-position (l = 1–16), pb_{il} the fixed effect of the interaction between the l-th position and the i-th treatment, e_{ijkl} the error effect of the ijkl-th plant, and Y_{ijkl} the length of the ijkl-th plant.

The seedlings were assessed in the order they were placed in the bags. When all seeds were assessed, the assessed position numbers l corresponded exactly to the position. However, the position of the excluded seedlings was not recorded. Hence, for analysis, empty positions were randomly inserted between filled positions so that the range of position numbers l was from 1 to 16 with some interspersed empty positions. This approximation was acceptable considering the low number of excluded seedlings and the absence of a treatment influence on this number. A variation of Model (4) considering a regression term (see Model (3)) was used but did not converge.

All analyses were performed with the MIXED procedure of the SAS software (Version 3.5, SAS Institute Inc., Cary, NC, USA). The treatment means were calculated and compared with the LSMEANS statement, using the Tukey-Kramer test for pairwise comparisons to control the family-wise Type I error rate. The Kenward-Roger method was used to determine the degrees of freedom of the denominator (option ddfm = kenwardroger) and to adjust standard errors. Normality of the residual errors was verified visually using residual plots. Variance homogeneity was checked visually for analyses of individual trials from plots of residuals versus predicted values. The GLIMMIX procedure of the SAS software was used for tests of covariance parameters (COVTEST statement).

The influence of the treatment factor on the frequency of excluded experimental material was investigated. Model (1) (individual trials) and Model (2) (meta-analyses) were applied on the number of discarded seeds and bags, assuming a generalised linear mixed model with a binomial distribution and a logit link (Piepho 1999). The GLIMMIX procedure was used with the verifications on normality and variance homogeneity described above.

Finally, the test power (Steel and Torrie 1980) was calculated in order to evaluate the efficiency of the bioassay to detect effects when they existed (protection regarding Type II error).

Results

Results from series A

The results of Series A (n = 76 trials) are presented in Table 3(a). Each trait in each trial was analysed with Model (1). The proportion r_s of trials that exhibited a significant treatment effect ($p < 0.05$; F-test) was constant for the hypocotyl traits over the growth period, but for the root traits it increased regularly (from 14.5% at day 2 to 38.2% at day 7). In the meta-analyses, $D_{1\mu l}$ exhibited significantly lower hypocotyl growth compared to Control at day 4 (−0.5%, $p = 0.04$, Tukey-Kramer-test) and to $D_{0.1\mu l}$ from day 3 to day 6 (at day 6: −0.6%, $p = 0.0006$). Concerning root growth, $D_{1\mu l}$ exhibited significantly lower growth compared to the Control at day 3 (−1.1%, $p = 0.0008$); this effect increased over time (at day 7: −2.4%, $p = 0.004$). $D_{0.1\mu l}$ also reduced root growth significantly at day 7 (−1.7%, $p = 0.03$). $D_{1\mu l}$ and $D_{0.1\mu l}$ differed significantly from day 2 to day 5.

Figure 3(a) details the results for root length at day 7 for all 76 trials. Compared to the Control, the $D_{1\mu l}$ treatment affected root length significantly ($p < 0.05$, Tukey-Kramer-test) in 27 trials (35.5%) and $D_{0.1\mu l}$ treatment in 18 trials (23.7%). This effect varied between −25.7% and +19.1% for $D_{1\mu l}$, and between −21.2% and +11.4% for $D_{0.1\mu l}$ (for all these 4 effects: $p < 0.001$). The treatments $D_{1\mu l}$ and $D_{0.1\mu l}$ differed significantly in 10 trials (13.2%).

Notably, the distribution of the 29 trials that exhibited a significant treatment effect was irregular. The treatment factor was significant in 5 of 6 trials (83.3%) during the first time period (I; July 2011 – Aug. 2011), in 3 of 37 trials (8.1%) during the second period (II; Aug. 2011 – May 2012), and in 21 of 33 trials (63.6%) during the third period (III; June 2012 – Jan. 2013). On average, the treatment $D_{1\mu l}$ increased root growth by 11.2% in period I and reduced it by 1.1% in period II and by 5.4% in period III, compared to Control. The treatment $D_{0.1\mu l}$ exhibited a similar pattern, but was lower in magnitude (+7.4%, −0.5% and −4.0% in periods I, II and III, respectively).

Results from series B

The effect of the HMP produced in 2012 was investigated in Series B, which was performed mainly over period III (33 of 38 trials; Table 3(b)). In the individual trials (Model 1), the proportion r_s of trials exhibiting a significant treatment effect tended to stay constant regarding hypocotyl growth, but for root growth it increased regularly over time (to 47.4% at day 7).

In the meta-analyses, the treatment factor did not affect the hypocotyl growth significantly. The $D_{1\mu l}$ treatment reduced the root growth significantly from day 3 (−1.4%, $p = 0.003$) to day 7 (−3.3%, $p = 0.001$) with a maximum at day 5 (−4.0%, $p < 0.0001$). The $D_{0.1\mu l}$ treatment also reduced root growth significantly from day 4 (−2.2%, $p = 0.0008$) to day 6 (−2.5%, $p = 0.02$) (Table 3(b)). The effects of $D_{1\mu l}$ and $D_{0.1\mu l}$ did not differ significantly, but a trend between the treatments was detected from day 4 to day 6 ($p < 0.09$).

The Figure 3(b) details the results of the 38 individual trials for the root length at day 7. Compared to Control, the $D_{1\mu l}$ treatment affected root length significantly ($p < 0.05$, Tukey-Kramer-test) in 17 trials (44.7%) and $D_{0.1\mu l}$ treatment in 12 trials (31.6%). Relative to Control, the average root length varied between −16.9% ($p < 0.0001$) and +14.2% ($p = 0.01$) for $D_{1\mu l}$ and between −14.7% ($p < 0.0001$) and +17.5% ($p = 0.002$) for $D_{0.1\mu l}$. These results were consistent with the Series A in the same period.

Table 3. Individual statistical analysis and meta-analysis for growth traits in series A, B and C. The values shown represent: n_s the number of individual trials with a significant treatment effect ($p < 0.05$, Wald F-test); $r_s = n_s/n$ (Series A: $n = 76$; B: $n = 38$; C: $n = 22$); p-value of the meta-analysis for the treatment factor; average length for the variants $D_{0.1\mu l}$, $D_{1\mu l}$ and C (mm); s.e.d. standard errors of differences (by variance heterogeneity: minimum and maximum). The variants (within columns) with no letter in common are significantly different (Tukey-Kramer-test, $p < 0.05$). The r_s-values above 5%, the p-values under 5% and the significant differences are shown in bold.

	Hypocotyl length (mm)				Root length (mm)					
	day 3	day 4	day 5	day 6	day 2	day 3	day 4	day 5	day 6	day 7
3a. Series A										
n_s	8	10	8	9	11	14	18	22	25	29
r_s	**10.5%**	**13.2%**	**10.5%**	**11.8%**	**14.5%**	**18.4%**	**23.7%**	**28.9%**	**32.9%**	**38.2%**
p-value	**0.03**	**0.0009**	**0.0005**	**0.0008**	**0.04**	**0.0002**	**0.0001**	**0.001**	**0.003**	**0.006**
Mean $D_{0.1\mu l}$	**17.55 a**	**32.65 a**	**45.41 a**	**55.05 a**	**12.50 a**	**30.96 a**	**50.38 a**	**62.90 a**	70.24 ab	**75.71 b**
Mean $D_{1\mu l}$	**17.41 b**	**32.37 b**	**45.08 b**	**54.73 b**	**12.40 b**	**30.62 b**	**49.74 b**	**62.19 b**	**69.55 b**	**75.12 b**
Mean C	17.47 ab	**32.55 a**	45.26 ab	54.86 ab	12.45 ab	**30.96 a**	**50.62 a**	**63.52 a**	**71.24 a**	**76.98 a**
s.e.d.	0.05	0.07	0.08	0.08	0.04	0.09	0.17–0.23	0.25–0.37	0.30–0.48	0.34–0.56
3b. Series B										
n_s	1	4	4	2	4	5	10	12	15	18
r_s	2.6%	**10.5%**	**10.5%**	**5.3%**	**10.5%**	**13.2%**	**26.3%**	**31.2%**	**39.5%**	**47.4%**
p-value	0.48	0.07	0.26	0.76	0.85	**0.004**	**< 0.0001**	**< 0.0001**	**0.001**	**0.007**
Mean $D_{0.1\mu l}$	17.21	32.29	44.72	54.48	13.66	31.67 ab	**48.94 b**	**59.92 b**	**68.69 b**	76.08 ab
Mean $D_{1\mu l}$	17.21	32.19	44.59	54.35	13.64	**31.50 b**	**48.41 b**	**59.07 b**	**67.77 b**	**75.22 b**
Mean C	17.27	32.41	44.76	54.40	13.64	**31.94 a**	**50.02 a**	**61.50 a**	**70.43 a**	**77.80 a**
s.e.d.	0.06	0.10	0.11	0.12	0.05	0.11	0.23–0.32	0.35–0.50	0.42–0.67	0.46–0.78
3c. Series C										
n_s	1	0	0	1	1	2	1	1	0	0
r_s	4.5%	0%	0%	4.5%	4.5%	**9.1%**	4.5%	4.5%	0%	0%
p-value	0.75	0.80	0.87	0.54	0.55	0.05	0.11	0.14	0.09	0.15
Mean C_1	17.74	32.51	44.44	53.94	13.19	32.08	50.50	61.71	69.75	76.21
Mean C_2	17.71	32.45	44.41	53.87	13.13	31.82	50.00	61.05	68.98	75.50
Mean C_3	17.69	32.50	44.47	54.03	13.17	32.09	50.38	61.33	69.26	75.75
s.e.d.	0.07	0.10	0.12	0.14	0.06	0.12	0.25	0.33	0.35	0.37

Notes: Every response variable of each trial in Series A, B and C was statistically evaluated with Model (1). The meta-analyses were performed with Model $(2)^{hom}$ with the following exceptions: Model $(2)^{het}$ for root length from day 4 to day 7 in Series A and for root length at day 4 in Series B, Model $(3)^{het}$ for root length from day 5 to day 7 in Series B.

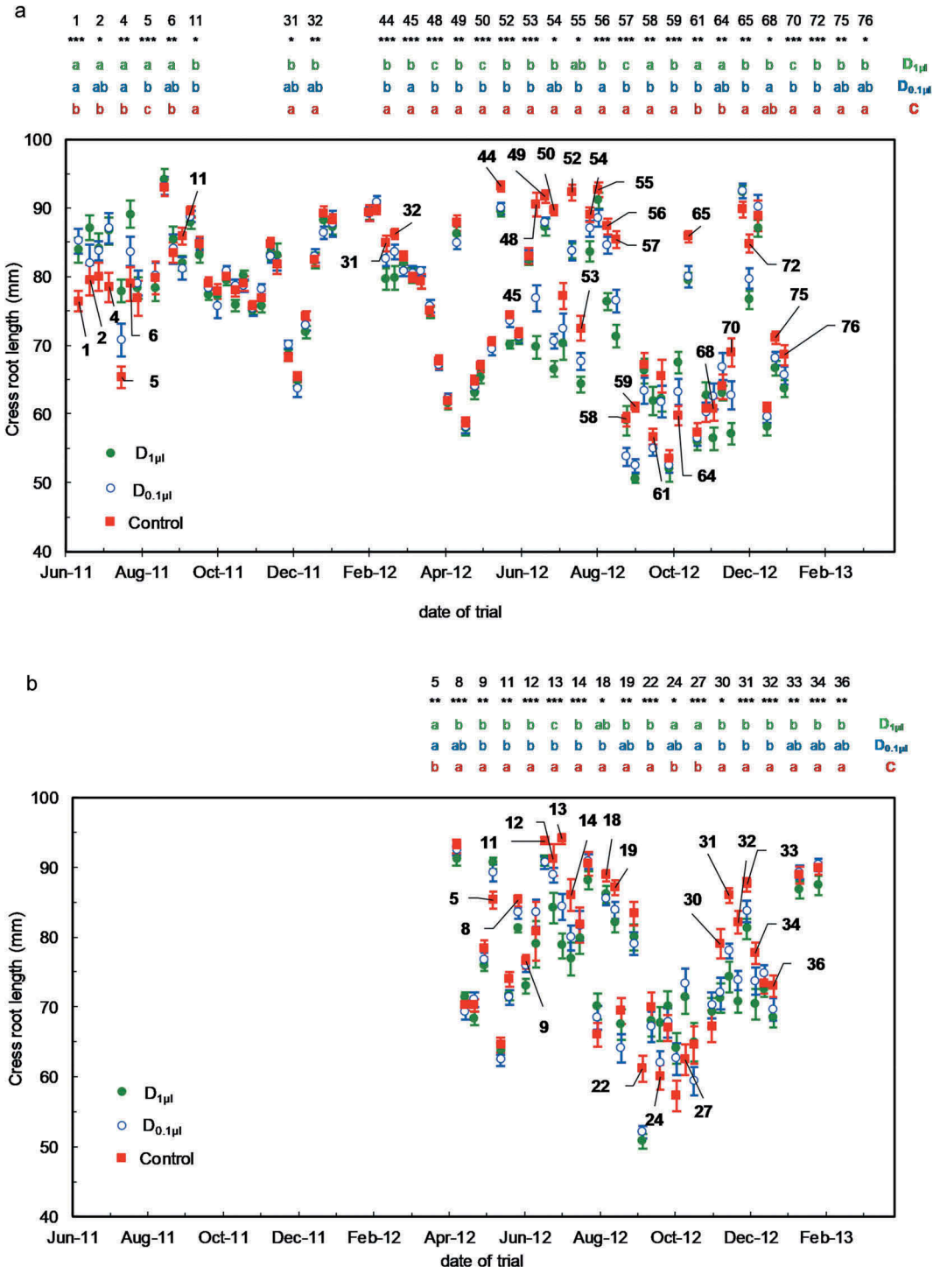

Figure 3. Root length of cress seedlings in dependence with the treatment factor (a) in 76 trials of the series A, and (b) in 38 trials of the series B. The treatments consisted of the addition of 1 µl HMP suspension ($D_{1µl}$, green filled circles), 0.1 µl HMP suspension ($D_{0.1µl}$, blue open circles), or 1 µl drilled well-water (Control, red squares). One point represents the average root length at day 7 (in mm) on the basis of 294 ± 12 seedlings, distributed over 20 bags. Error bars represent ± standard error. The statistical analysis of trials with significant results (trial number indicated) is reported in detail: (i) Asterisks indicate the *p*-value for the treatment factor by a Wald F-test ($n = 20$): * ($0.01 < p < 0.05$), ** ($0.001 < p < 0.01$), *** ($p < 0.001$). (ii) The treatments with no letters in common differ significantly by a Tukey-Kramer-test ($p < 0.05$, $n = 20$).

Results from series C: negative control trials

The 22 negative control trials of Series C tested the false positive rate of the bioassay (parameter r_s in Table 3(c)). This rate was by 9.1% for root length at day 3, and under 5% for all other traits. In the meta-analyses, the treatment factor was not significant for any trait. For root length at day 7, the observed false positive rate was null (Supplemental Figure A).

Stabilising effect: comparison of models 2 and 3

In the individual trials, the significant effects of the HMP treatment on root length were either positive or negative. Considering the root length at day 7, a linear regression of HMP means versus Control means revealed slopes smaller than 1 for both treatments in Series A ($D_{1\mu l}$: 0.87; $D_{0.1\mu l}$: 0.93; Figure 4(a)) and in Series B ($D_{1\mu l}$: 0.73; $D_{0.1\mu l}$: 0.87; Figure 4(b)). These observations suggested a stabilising effect.

The inference for this regression was not straightforward because both variables were subject to estimation error and heterogeneity of the interaction variance can lead to spurious departures from a unit slope. Instead, statistical models in which the experimental conditions that influenced the treatments (factor-analytic variance-covariance structure), or not, were compared (Piepho 1997b). This comparison provided significant evidence of lower variances for HMP treatments in both series, implying a stabilising effect ($p < 0.001$). Furthermore, a stabilising response to changing experimental conditions (regulating effect) was indicated for $D_{1\mu l}$ treatment in series B ($p = 0.02$) as per the estimate of slopes c_i.

The details of this comparison of statistical models were: The hypothesis of a stabilising effect was tested with two parameters: (a) the interaction variance σ^2 and (b) the slope c_i in Model (3). For (a), the interaction variance was assessed by considering the homogeneity (hom) or heterogeneity (het) in the models (2) and (3), and for (b), the hypothesis of a stabilising response to changing experimental conditions corresponds to the slope c_i in Model (3). In Model (2), all treatments responded equally to changing experimental conditions and in Model (3) they responded differentially. Likelihood ratio tests between $(2)^{hom}$, $(2)^{het}$, $(3)^{hom}$ and $(3)^{het}$ were performed. The results from fitting all four model variations are presented in Table 4 (parameter is the root growth at day 7). The relevant estimates of parameters for Model $(3)^{het}$ (Eberhart-Russell model) are given in Table 5.

For the first parameter (a), by comparing $(2)^{hom}$ with $(2)^{het}$ and $(3)^{hom}$ with $(3)^{het}$, the likelihood ratio test significantly assessed the heterogeneity of variance in Series A and B ($p < 0.001$). In both Series, the variances of $D_{1\mu l}$ and $D_{0.1\mu l}$ in $(2)^{het}$ and $(3)^{het}$ were highly significantly lower than the variance of the Control (for $(3)^{het}$: $p < 0.001$, Table 5). Hence, the stabilising effect of $D_{1\mu l}$ and $D_{0.1\mu l}$ was established.

For the second parameter (b), assuming heterogeneity of variance, the Models $(2)^{het}$ and $(3)^{het}$ were compared. The likelihood test was significant for Series B ($p = 0.01$), but not for Series A ($p = 0.33$). The hypothesis H_0 that the slopes c_i are equal for the three treatments was therefore rejected only for Series B. In Series B, the regression slope of the treatment $D_{1\mu l}$ at 0.92 was the lowest, indicating a stable response to changing experimental conditions (Table 5). It differed significantly from $D_{0.1\mu l}$ (1.05), but not from the Control (1.03). Notably, it differed significantly from the Control for root length at day 5 ($p = 0.02$, data not presented).

Influence of the position of the seedling in the bag

The drop application induced a non-uniform dispersion of the HMP suspension in the bag. To study the influence of this dispersion, the relationship between the position of the seedlings and the

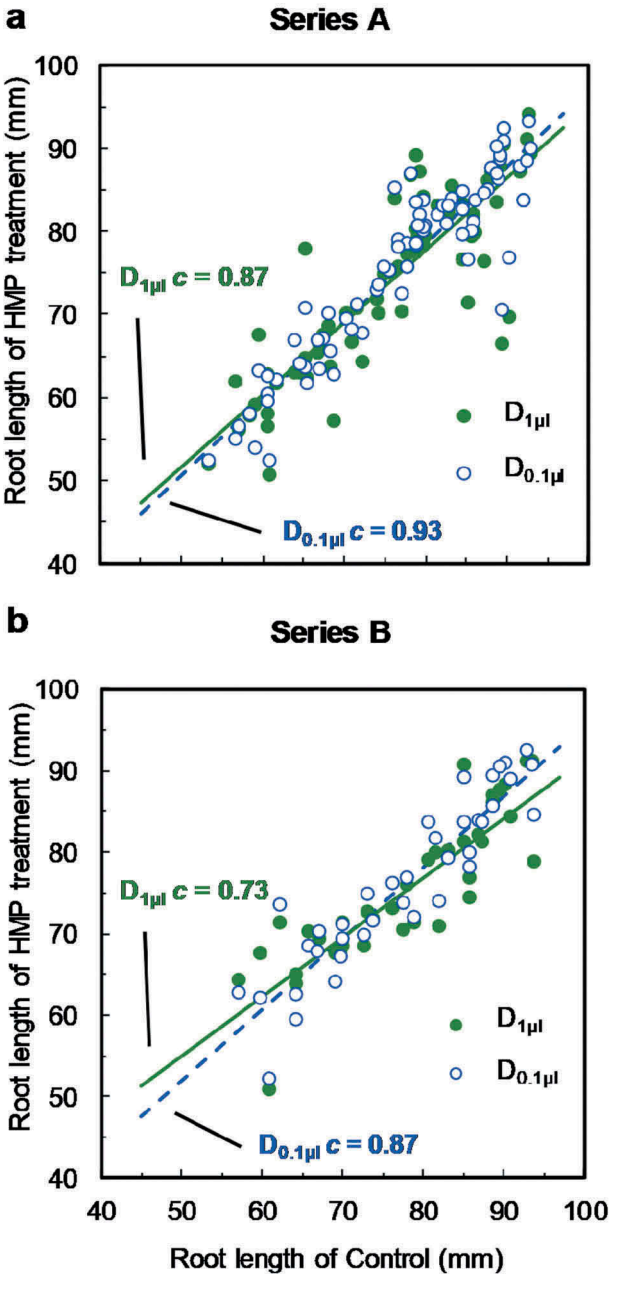

Figure 4. Linear regressions of $D_{1\mu l}$ or $D_{0.1\mu l}$ means versus Control means in Series A (a) and Series B (b). One point ($D_{1\mu l}$: green filled circle, $D_{0.1\mu l}$: blue open circle) represents the average root length at day 7 (in mm) in one individual trial on the basis of 294 ± 12 seedlings, distributed over 20 bags. The linear regression with linear coefficient *c* is represented for the treatments $D_{1\mu l}$ (green, full) and $D_{0.1\mu l}$ (blue, dashed).

treatment factor was statistically analysed with Model (4) for root length at day 7 (Table 6, Figure 5). In all series, the factor *position* was highly significant ($p < 0.0001$), mainly because of a growth increase at the borders of the bags (positions 1 and 16, Figure 5). The interaction between the

Table 4. Goodness-of-fit parameters for different statistic models. The data represent twice the likelihood-coefficient ($-2\ LL_R$) and the Akaike information criterion (AIC) for models (2) and (3) assuming variance homogeneity or heterogeneity. Trait is the root length at day 7.

	Series A		Series B	
Model	$-2\ LL_R$ [1]	AIC[2]	$-2\ LL_R$	AIC
$(2)^{hom}$	29,366.3	29,374.3	15,672.4	15,680.4
$(2)^{het}$	**29,322.1**	*29,332.1*	15,652.9	15,662.9
$(3)^{hom}$	29,365.7	29,377.7	15,666.2	15,678.2
$(3)^{het}$	29,319.9	29,333.9	**15,643.9**	*15,657.9*

Notes: [1] The final analyses were performed with the models in bold.[2] Smaller is better (in italics)
Between Models $(2)^{hom}$ and $(2)^{het}$ and between Models $(3)^{hom}$ and $(3)^{het}$, the differences of $-2\ LL_R$ above 13.82 are significant at $\alpha = 0.001$ (χ^2 distribution, df = 2). Between Models $(2)^{hom}$ and $(3)^{hom}$ and between Models $(2)^{het}$ and $(3)^{het}$, the differences above 5.99 are significant at $\alpha = 0.05$ (χ^2 distribution, df = 2).

Table 5. Parameter estimates for variance – covariance structure of the effects of HMP on root length at day 7. The values shown represent: the interaction variance (σ^2) and the regression slope (c). The parameter estimates with no letters in common differ significantly by a χ^2 distribution (c: $p < 0.01$; σ^2: $p < 0.003$).

Parameter	Treatment	Series A	Series B
σ^2	$D_{0.1\mu l}$	0 a	0 a
	$D_{1\mu l}$	5.6 b	1.4 a
	Control	15.1 c	15.8 b
c	$D_{0.1\mu l}$	1.03	1.05 b
	$D_{1\mu l}$	1.01	0.92 a
	Control	0.96	1.03 ab

Notes: The variance – covariance structure is fitted with Model $(3)^{het}$. To test the null hypothesis (H_0: $c_1 = c_2 = c_3 = 1$) the main effect of the trial (t_j) was assumed to be random and have a mean of zero, which accounted for the fact that trial means were subject to error. This induced a factor-analytic covariance structure (Piepho 1997b) and slight re-parameterisation (because it is used in the software SAS), setting $\lambda_i u_j = c_i t_j$, where u_j has a mean equal to zero and a variance equal to one. The factor-analytic structure has parameters λ_i (i = 1, 2, 3), from which the slopes c_i in Model $(3)^{het}$ can be computed as $c_i = \lambda_i/\lambda_.$, where $\lambda_.$ is the mean of the λ_i (i = 1, 2, 3).

Table 6. *p*-values of fixed effects in model (4) for the series A, B and C. Trait is the root length at day 7.

Series	Treatment	Position	Treatment*Position
A	0.001	< 0.0001	0.22
B	0.001	< 0.0001	0.84
C	0.16	< 0.0001	0.07

position and the HMP treatment was not significant in Series A ($p = 0.22$) and B ($p = 0.84$). Therefore, the results revealed no indication of a relationship between the position and the treatment factor. No relationship was found for the other root traits (day 2 to day 6) in series A and B as well (results not detailed).

In Series C, a slight interaction between treatment and position was indicated, though this was not significant ($p = 0.07$). The examination of the interaction effects did not reveal a regular influence of treatments but confirmed the influence of the borders of the bag.

Discarded seeds and bags

In all series, 96 of 8,160 bags (1.2%) and 9,166 seedlings from the remaining bags (7.0%) were discarded (Supplemental Table A). The influence of treatment on proportion of excluded seedlings was statistically evaluated for each trial. The treatment factor was significant in 2 trials in Series A (2.6% of all trials), 2 in Series B (5.2%) and 0 in Series C (0%).

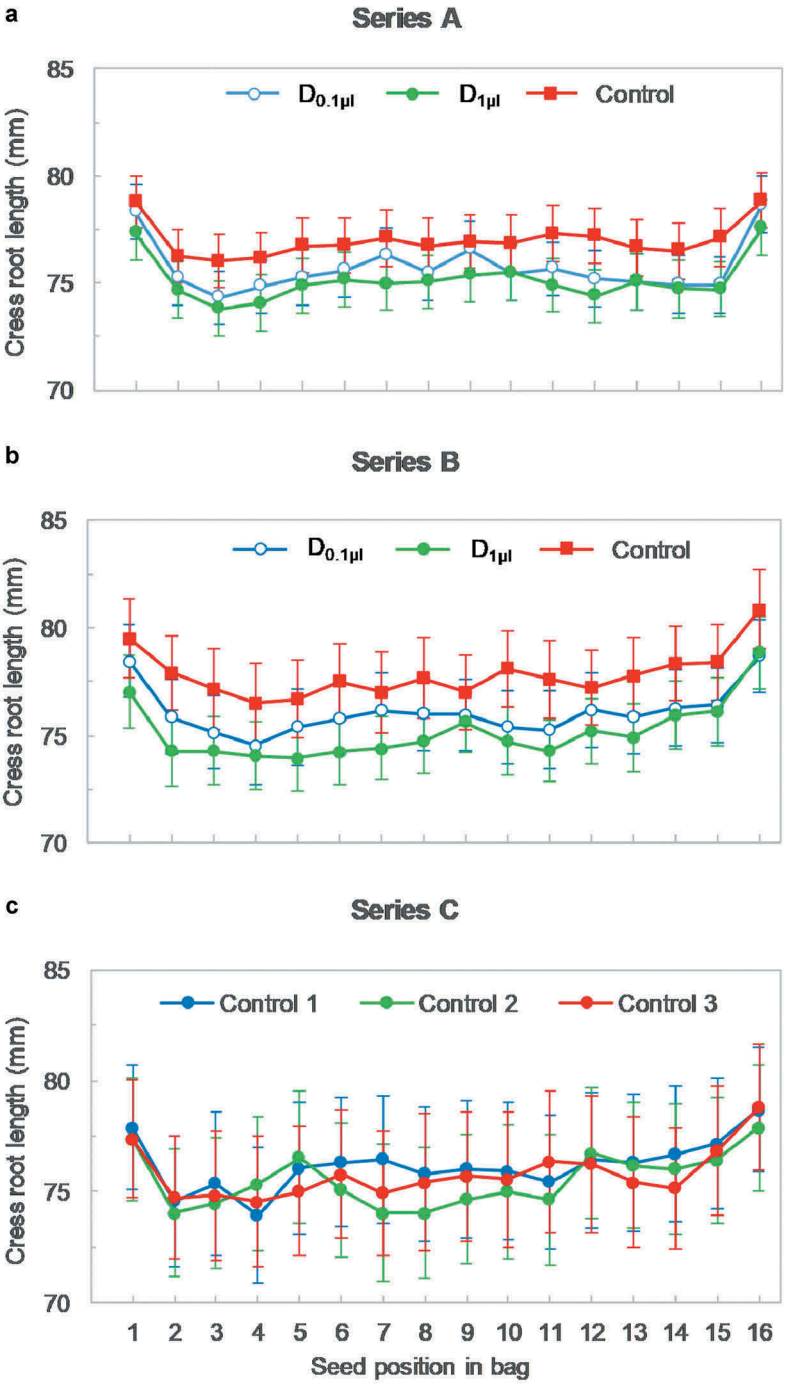

Figure 5. Root length at day 7 of cress seedlings in dependence with the seedling position in bag and the treatment factor in series A (a), B (b) and C (c). One point represents the average root length at day 7 (in mm) on the basis of 1407 ± 14 seedlings in series A, 705 ± 11 in series B and 403 ± 7 in series C. Error bars represent ± standard error.

In the meta-analyses, the treatment factor did not significantly affect the number of excluded bags (Series A: $p = 0.28$; B: $p = 0.16$; C: $p = 0.10$) and of excluded seedlings (Series A: $p = 0.25$; B: $p = 0.71$; C: $p = 0.60$).

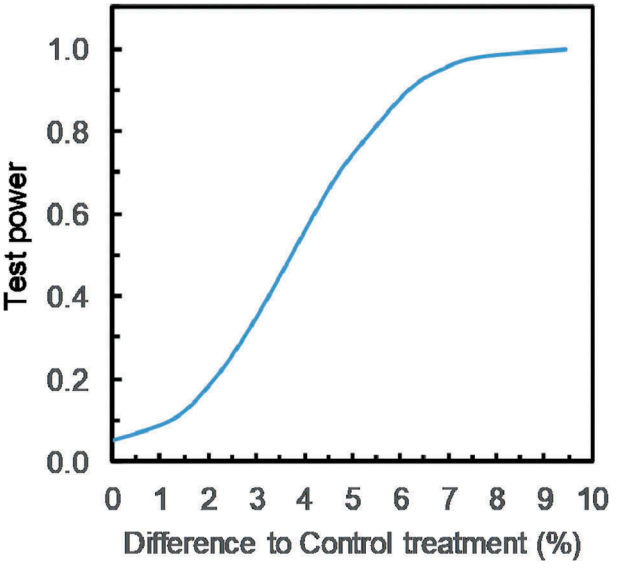

Figure 6. Test power versus difference to control. The calculation is based on a repetition number of 20 bags, a level of significance of 0.05 and a standard error of difference of 1.91% (relative to the mean; median over all trials). Trait is the root length at day 7.

Statistical power of the test

To evaluate the efficiency of the bioassay to detect effects when they existed (protection regarding Type II error), the statistical power of the test was calculated (Figure 6). A growth difference over 5.3% had a probability of 80% to be detected as significant, and a difference over 6.2% a probability of 90% (test power of 0.8 and 0.9, respectively).

Discussion

Plant growth response

In the present study, the effects of low doses of HMP on plant growth were investigated. The main effects were observed for root growth. For the root length at day 7, the treatment $D_{1\mu l}$ differed significantly from the Control in Series A and B ($p = 0.004$ and $p = 0.001$, respectively). Significant effects of $D_{1\mu l}$ were detected in 35.5% and 44.7% of the trials of Series A and B, respectively. Effects of $D_{0.1\mu l}$ were similar (Series A: $p = 0.03$; Series B: $p = 0.06$; significant effects in 23.7% and 31.6% of the trials of Series A and B). For this trait, the observed false positive rate in the systematic negative control trials (Series C) was null, thereby endorsing the reliability of the investigated bioassay. It was concluded that the effect of HMP on the early root development verified the working hypothesis, showing the sensitivity of plant growth to HMP at an early stage.

Furthermore, the results of Series A and B were consistent, indicating that the root growth response was independent of the HMP investigated. The two HMPs differed by their production year (2010 and 2012). Hence, an aging of the HMP under 3 years did not appear to influence its bioactivity.

Influence of the dose

In the meta-analyses of different traits, the $D_{0.1\mu l}$ and $D_{1\mu l}$ treatments differed significantly in Series A (hypocotyl length at day 3–6 and root length at day 2–5) and nearly significantly in Series B ($p < 0.09$, root length at day 4–6). For root length at day 7, the differences were not significant.

Concerning the individual trials, the effect of $D_{0.1\mu l}$ for this trait was typically lower than that of $D_{1\mu l}$, but only few significant differences were reported (Series A: in 10 trials, 13.2%; Series B: in 1 trial, 2.6%). It seemed that the test power was insufficient to detect the differences.

Stability over time

The results showed a strong variability of the root growth level over time. As described in other bioassays (Scherr et al. 2008), sources of natural variability are seasonal conditions and endogenous periodicities of the test organism. This variability was introduced by the trial conditions as well: varying natural light and room temperature (during the daily marking of the length) and the use of well-water as cultivation medium. These sources of variability can also be considered to explain the high fluctuations of the root growth responses to HMP.

Stabilising effect of HMP

Although the HMP effects on the plant growth were in general small and fluctuating, a stabilising effect was statistically established by smaller interaction variances of $D_{1\mu l}$ and $D_{0.1\mu l}$ compared to the Control for both Series A and B. Furthermore, a stabilising effect depending on environmental conditions was indicated for $D_{1\mu l}$ in series B by a smaller slope c_i.

Past studies described a similar pattern of action. Reporting on laboratory studies, Dewes and Ahrens (1990) described a stabilising effect of HMP on biological activity in soil, depending on the availability of the organic substances. Raupp and König (1996) analysed the results of 28 field plot and pot trials and also suggested a stabilising effect for BD preparations used in combination. The same authors reported a similar trend in another long-term field trial, but the results of this trial were later ascribed to soil heterogeneity (Heitkamp et al. 2011). Goldstein and Barber (2005) described a stabilising effect from a 6-year field trial. It is to be noted that the regression analyses of Goldstein and Barber (2005) and Raupp and König (1996) were biased because both variables (response for treatment and observed control value) were subject to estimation error (Fuller 1987). Hence, only the basic experimental results of these studies were considered here, but not the regression. In summary, these past results supported the results from this study that the HMP bioactivity exerted a stabilising pattern of action.

Practical relevance

This bioactivity may induce an increased resilience of the agricultural system, which would be of great importance in practice to secure crop yield stability. However, the relative differences to the Control were small. Indeed, the magnitude of detectable effects was at 5.3% (by a test power of 80%). In the meta-analyses, significant differences as low as 1.1% were detected, which raised the question about the practical relevance.

Generally, the transfer of results obtained under laboratory conditions to natural conditions is challenging. Moreover, the present effects on growth were observed after only one week and their long-term evolution is unknown. Indeed, small differences at early stage can be compensated and disappear thereafter, or, on the contrary, lead to remarkable variations at harvest. Only field experiments could give a satisfactory answer on the practical relevance. Therefore, they would be the logical follow-up of the present investigations.

Influence of the drop application

The application of a drop of the HMP suspension mimicked the BD practice, but induced a non-uniform dispersion of the HMP suspension in the bag. However, no influence on the effect of the HMP was reported; the responses of the seedlings at the borders or in the middle were the same,

although they received different doses of the HMP. Moreover, this stability was verified at all root growth stages.

Therefore, this result indicated a dose-independent bioactivity of the HMP. However, a dose-independent effect apparently contradicted the differences between $D_{0.1\mu l}$ and $D_{1\mu l}$ as indicated above. Further conclusions could not be drawn because of the lack of knowledge about the origin of the bioactivity of HMP. More investigations are necessary to clarify the question of the dose-response relationship of the HMP.

Future test development

The test power was at 0.8 for a growth difference of 5.3% and it appeared that this test power was insufficient. Hence, it should be increased to detect effects of lower range. A first option would be to increase the number of bags. With 40 instead of 20 bags, the test power would theoretically be at 0.8 for a difference of 3.7%. A second option would be to reduce the overall variation, requiring the control of the main test factors and the standardisation of trial conditions. The reduction of the overall variation would improve the bioassay stability in the time as well. The present results showed that the bioassay stability was a main issue.

Origin of the bioactivity of HMP

The present results assessed the bioactivity of HMP at low doses. Spaccini et al. (2012) revealed the potential of bioactivity of HMP on the basis of molecular analyses. These analyses exposed a structure with lignin aromatic derivatives, polysaccharides, and alkyl compounds as the main components. The content of labile molecules and aromatic lignin derivatives tended to be higher in HMP than in common mature compost, indicating that HMP may be potentially more labile in soil and more bioactive toward plant growth. This high content could be due to the slow maturation of HMP that occurs in winter and in soil (Spaccini et al. 2012).

However, HMP is a complex chemical and microbiological mixture and its bioactivity cannot be straightforwardly explained. A relationship may be assumed with the bioactivity at low doses of humic substances that has gained attention for agricultural applications (Rose et al. 2014; Canellas et al. 2015; Nardi et al. 2016). In particular, effects of lignosulfonate-humate on cress root length have been detected at a concentration (0.5 mg C l^{-1}; Ertani et al. 2011) comparable to the roughly estimated concentration of the HMP in the present study. The bioactivity of humic substances has mainly been recognised as enhancing root nutrition, but stress response modulation has been described as well (Rose et al. 2014; Du Jardin 2015). This modulation may correspond to the stabilising effect of the HMP indicated in the present work. Furthermore, the role of environmental conditions in the sensitivity of plant growth response to humic substances is prominent and multi-faceted (Rose et al. 2014). The present results assessed this sensitivity with regards to the HMP.

Hormonal effects of humic substances have been described as well, but it is not clear if this effect is due to entrapped hormonal compounds, to hormone-like functional groups, or to stimulation of hormone-producing microorganisms (Du Jardin 2015). The same uncertainty applies for the HMP. Giannattasio et al. (2013) exposed the potential bioactivity of HMP as a soil stimulant of microbiological and enzymatic properties. They reported auxin-like activity by higher concentrations of HMP (1 g HMP l^{-1} corresponding to 0.03 ppm of indol-acetic acid) than in the present study. Furthermore, Radha and Rao (2014) reported the presence of auxin-producing bacterial strains in HMP that could enlighten a phytohormone-based activity as well. However, Botelho et al. (2015) only detected the presence of cytokinin isopenthyl adenosine in HMP, at very low concentration compared to the concentration used in commercial products. They did not identify isopenthyl adenine, IAA acid or abscisic acid.

The chemical and microbiological complexity of HMP may also result in non-linear and cross-interrelated processes as assumed for humic substances (Canellas and Olivares 2014). Yakhin et al. (2017) considered that the bioactivity of biostimulants may be not ascribable to a particular constituent, but to the complex of its constituents as a whole (emergent property). This direction of thought could apply for the HMP as well.

Conclusions

The conclusions were as follows. (1) The cress root growth, at the early growth stage, was highly sensitive to effects of HMP. (2) The effect of HMP was strongly dependent on time, but stable within periods of many months. (3) A stabilising pattern of action was significant, indicating the potential to increase the resilience of the agricultural system in practice. (4) The effect was not affected by the non-uniform dispersion of the HMP suspension, but differences between two low doses were observed. Hence, the working hypothesis that HMP affects the root development during the early germination process was verified, and new insights on the bioactivity of the HMP were gained. The bioassay design showed promise for investigating this bioactivity. Further investigations are needed to standardise the trial conditions and to improve the stability and power of the bioassay.

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Disclosure statement

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