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# Interactions between abiotic factors and the bioactivity of biodynamic horn manure on the growth of garden cress (*Lepidium sativum* L.) in a bioassay

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## Abstract

**Background:** The use of biostimulants like humic substances is a promising innovative approach in agriculture to activate and sustain physiological plant processes. The development of specific bioassays is required to study their bioactivity in laboratory conditions. In previous investigations, a soil-less bioassay with cress seedlings (*Lepidium sativum* L.) was developed for a biostimulant used in the biodynamic agriculture, the horn-manure preparation (HMP), a fermented cow manure sprayed at low concentrations onto fields. Objectives of the present study were to refine the bioassay by investigating the interactions between the HMP bioactivity and the test factors (i) water volume, (ii) gravistimulation, and (iii) exposure to fluorescent light.

**Results:** The interactions between the test factors and the HMP treatment were significant in all series ($p < 0.05$, Wald *F*-test). Water overdose and gravitropic stress reduced root growth (down to − 24.2% and − 19.9%, respectively, $p < 0.0001$, Tukey–Kramer test). The HMP treatment partly compensated these effects by enhancing root growth by (i) water overdose (up to + 4.3%, $p = 0.048$, $n = 4$), and (ii) gravitropic stress (up to + 9.5%, $p = 0.0004$, $n = 8$). (iii) Furthermore, under the combined stress factors, fluorescent light exposure enhanced the HMP enhancing effect (up to + 12.3%, $p = 0.007$, $n = 6$).

**Conclusions:** The HMP bioactivity appeared to consist of a compensatory mode of action regarding the stress factors water overdose and gravistimulation, and a synergetic interaction with fluorescent light exposure. The HMP seems to interact with the plant sensory systems, likely stimulating the plant's adaptability to its environment by increasing self-regulating processes. The bioassay sensitivity was successfully increased by integrating these interactions in the experimental set-up and adjusting the growth environment. This approach can be used to adjust the bioassay to other biostimulants.

**Keywords:** *Lepidium sativum* L., Humic substances, Biodynamic agriculture, Biostimulant, Bovine manure, Low dose effect, Bioassay, Gravistimulation, Phototropism

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## Background

Agricultural production faces important ecological constraints and growing social demands for sustainability and food quality, increasing the need for innovative agronomical approaches. One promising approach is the use of biostimulants like humic substances (HS) to activate physiological plant processes [1–4]. Indeed, HS not only influence the soil properties, but, at low doses, also enhance plant germination and regulate growth stimulation [5].

Recommendations for using HS in agriculture are often directed at alleviating environmentally stressful conditions such as drought, salinity, chilling, oxidative stress, heavy metal toxicity, or nutrient deficiency [1, 2, 6]. Nevertheless, the effectiveness of HS under stressful conditions has been rarely investigated at low application rates [7]. Therefore, the development of specific bioassays for biostimulants is of interest to assess their bioactivity in stress conditions.

In this regard, Baumgartner et al. [8] used for their preclinical investigations a soil-less bioassay with interesting features. As test organisms, cress seedlings (*Lepidium sativum* L.) were cultivated in hanging LD-PE plastic bags. This simple and quick bioassay has many interesting properties including high number of replications, easy handling and non-intrusive observation of root growth. This is particularly advantageous, because the root system is highly sensitive to bioactive substances, but it is usually less studied due to limited accessibility [9].

The intention of the present research was to adapt this bioassay for a HS biostimulant used in the biodynamic agriculture, the horn manure preparation (HMP). In agricultural practice, this humus mixture obtained from fermented cow manure is applied onto fields by concentrations of 120 to 300 g $ha^{-1}$. Giannattasio et al. [10] have estimated that HMP concentration in soil water amounts to 0.4 mg HMP $L^{-1}$. This concentration is very low, but bioactivity of HS had been reported at similar concentrations of 0.5 mg C $L^{-1}$ in laboratory conditions [11]. Analyses of molecular structure and enzymatic activity have indicated the potential of a high bioactivity of HMP [10] [12]. This bioactivity has been assessed in some incubation studies by laboratory conditions [13, 14] and on field trials (for example, recently [15–18]).

In a former own study, a modified version of this bioassay was tested with long-term trial series [19]. Results showed that HMP, at doses comparable to the biodynamic practice, significantly influenced root growth at early growth stages. A stabilizing pattern of action was statistically established by demonstrating smaller interaction variances of the HMP treatments compared to the Control. Furthermore, this stabilizing effect partially depended on experimental conditions, such that growth was enhanced in trials with below-average growth and reduced in trials with above-average growth.

Further refinement of the bioassay can be achieved by adjusting the growth conditions, notably by integrating stress factors. Indeed, the underlying ideas of many test designs are to use the interaction between the investigated substance and the plant reaction to a stress. For example, in the bioassay of interest, Baumgartner et al. [8] applied colchicine to the cress seedlings in order to induce a stress reaction suitable for investigating the effectiveness of a pharmaceutical product. However, if a stress reaction underlies the design of a bioassay, a main challenge is to set an appropriate stress level [20]. At high stress, test organisms are severely weakened and the investigated substance cannot counteract acute stress symptoms. Conversely, at a low stress level, impairment of the test organisms is not sufficient. Therefore, stress level and plant vitality should be appropriately balanced to allow measurement of the resilience process [20].

However, instead of a single stress factor, a combination of different stimulations can be considered as well. Indeed, this approach could allow more flexibility by designing the bioassay. The search of such a combination was the goal of the present study. Preliminary trials were conducted to investigate the interactions of the HMP bioactivity and different abiotic factors. The unpublished results suggested the hypothesis of the present study that the HMP bioactivity interacts with (1) water volume, (2) gravity stimulation and (3) light exposure. In the present research, three series of experiments were performed in order to test the hypotheses (1), (2) and (3) stated above and to find the test conditions optimizing the sensitivity of the bioassay.

## Materials and methods

### Material: HMP suspension and cress seeds

The HMP and HMP suspension were produced at the research site Landbauschule Dottenfelderhof e.V. (Bad Vilbel, Germany) according to the biodynamic criteria [21]. Cow manure was collected and placed in cow horns, which were then buried during winter and unearthed in spring 2015. The HMP was the 'humus mixture' that resulted from this fermentation. For use, a suspension of 21 g of HMP in 7 l water collected from a drilled well at the research site with was stirred by hand during one hour. This water suspension was produced anew when setting up each independent trial.

The cress seeds (Bingenheimer Saatgut AG Echzell, Germany) were organically certified. Seeds were controlled and removed if damaged or deviating in size, shape or colour.

### Experimental procedure of the bioassay

The experimental procedure was described thoroughly in [19]. It was based on the procedure presented by Baumgartner et al. [8]. Cress seedlings were cultivated in suspended LD-PE bags (Minigrip ® 120 × 170 mm, Inteplast Group, USA) in a light-isolated incubator (KB 720, Binder GmbH, Germany) at 19 °C. Chromatography paper (FN 1, Sartorius AG, Germany) was introduced into each bag. The bags were filled with well-water collected from the research facility as cultivation medium (volume was a varying factor, see next section). 16 cress seeds were introduced into the bags and aligned on the soaked chromatography paper 10 cm above the bottom of the bag (Fig. 1). The *dose* treatment consisted of the application of a drop on the chromatography paper in the middle of the bag with a microliter syringe (Acura 825, Socorex Isba S.A., Switzerland), 5 to 7 h after the seeds' introduction. The drop consisted of either (i) 1 µl well-water (Control, *C*), (ii) 0.1 µl HMP suspension ($D_{0.1\mu l}$) or (iii) 1 µl HMP suspension ($D_{1\mu l}$). The application of the HMP suspension as one drop at early stage of seed imbibition mimicked biodynamic practice (dispersion in droplets on the field at sowing).

Root and hypocotyl growth were marked daily with a point on the bags. During this operation, the bags were taken from the incubator and placed on a table at room temperature (laying time and light were varying factors, see next section). Trials were terminated after 6, 7 or 8 days, depending on the growth dynamic. The bags were then photographed and the points marked on the bags were analysed via image analysis software (Sigma Scan Pro 5.0, SPSS Inc., USA) to assess the daily growth of root and hypocotyl.

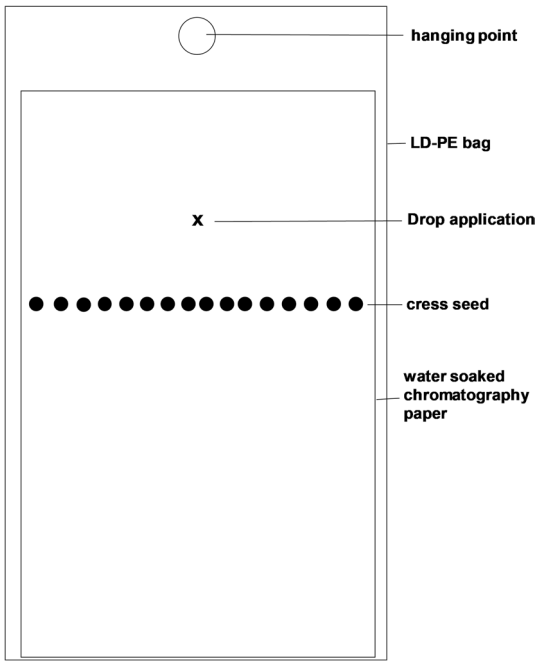


**Fig. 1** Schematic layout of one bag

In all steps, *dose* treatments were blinded by using coded bags. An exception is the drop application phase, because this required the addition of two drop volumes (0.1 µl and 1 µl) that were necessarily different. Samples were decoded at the very end of the trial, after all length measurements were accomplished.

During the marking operation, seedlings with skewed or retarded growth were visually identified and not considered. Therefore, exclusion of experimental material was blinded as well. A bag was excluded from the final analyses if more than 7 of the 16 seeds had been discarded.

### Trial series

Three 2-factorial series of trials (W, G, and L) were conducted to investigate the interaction between the HMP bioactivity (*dose* factor) and the following *test* factors (Table 1):

- *Water volume* factor in Series W. It corresponded to the quantity of water poured in the bags with three levels: 4 ml, 5 ml, and 6 ml.
- *Laying time* factor in Series G. It corresponded to the period the bags were laid on table during the daily growth marking (see experimental procedure). During this laying time, the plant gravity sensing system was disturbed. Levels were 1 min, 20 min, 40 min and 60 min.
- *Light* factor in Series L. It corresponded to the light the seedlings were exposed to during the daily growth marking (see experimental procedure). Five levels were considered: natural light (NL) and fluorescent light (FL) at four illuminances (FL 100 Lux, FL 500 Lux, FL 1000 Lux and FL 1500 Lux). The illuminances (light meter TES-1336A, TES Electrical Electronic Corp., Taiwan) were adjusted by varying manually the distance between a white fluorescent tube (Lumeno® 28 W, Traderia GmbH, Germany) and the table. For the FL variants, the room was in the dark and the exposure time was 1 min. For the NL variant, the room was illuminated with sun light. The uncontrolled illuminance fluctuated between 100 Lux and 1000 Lux.

In Series W and G, the *dose* factor had three levels (*C*, $D_{0.1\mu l}$ and $D_{1\mu l}$), in Series L two levels (*C* and $D_{1\mu l}$).

The randomization layout of each series was a split-plot design with *test* factor as whole-plot factor and *dose* as

**Table 1 Description of Series W, G and L**

| Series | Experimental period | Trial number | Fixed test conditions | Varying test conditions | | *Dose* levels |
|---|---|---|---|---|---|---|
| | | | | Test factor | Levels | |
| W | May–June 2015 | 4 | Laying time: 20 min<br>Light: natural light | *Water volume* | 4 ml<br>5 ml<br>6 ml | *C*<br>$D_{0.1\mu l}$<br>$D_{1\mu l}$ |
| G | June–August 2015 | 8 | Water volume: 6 ml<br>Light: natural light | *Laying time* | 1 min<br>20 min<br>40 min<br>60 min | *C*<br>$D_{0.1\mu l}$<br>$D_{1\mu l}$ |
| L | September–November 2015 | 6 | Water volume: 6 ml<br>Laying time: 40 min | *Light* | FL 100 Lux<br>FL 500 Lux<br>FL 1000 Lux<br>FL 1500 Lux<br>Natural light | *C*<br>$D_{1\mu l}$ |

Trial number acts as time replications, test factor as whole factor, and dose as split-plot factor

sub-plot factor (Fig. 2). In each series, one time replication consisted of one independent trial (Series W: 4 trials; Series G: 8; Series L: 6). The trials were conducted sequentially in the time. In each trial, one whole-plot consisted of one independent hanger, one for each levels of the *test* factor (Series W: 3 hangers; Series G: 4; Series L: 5). On each hanger, one sub-plot consisted of one suspended bag with 16 seeds (20 bags per *dose* level). Two randomization levels were applied (1) by varying daily the hanger positions in the incubator at random (*test* factor), and (2) by assigning the bag positions on hanger according to a randomized complete block design (*dose* factor).

The experimental set-up aimed at investigating the combination of gravitropic stress and water overdose in each series. Accordingly, as described in Table 1, the fixed test conditions were defined in order to apply gravitropic stress in Series W (fixed laying time: 20 min), water overdose in Series G (fixed water volume: 6 ml), and both stress factors in Series L (laying time: 40 min; water volume: 6 ml).

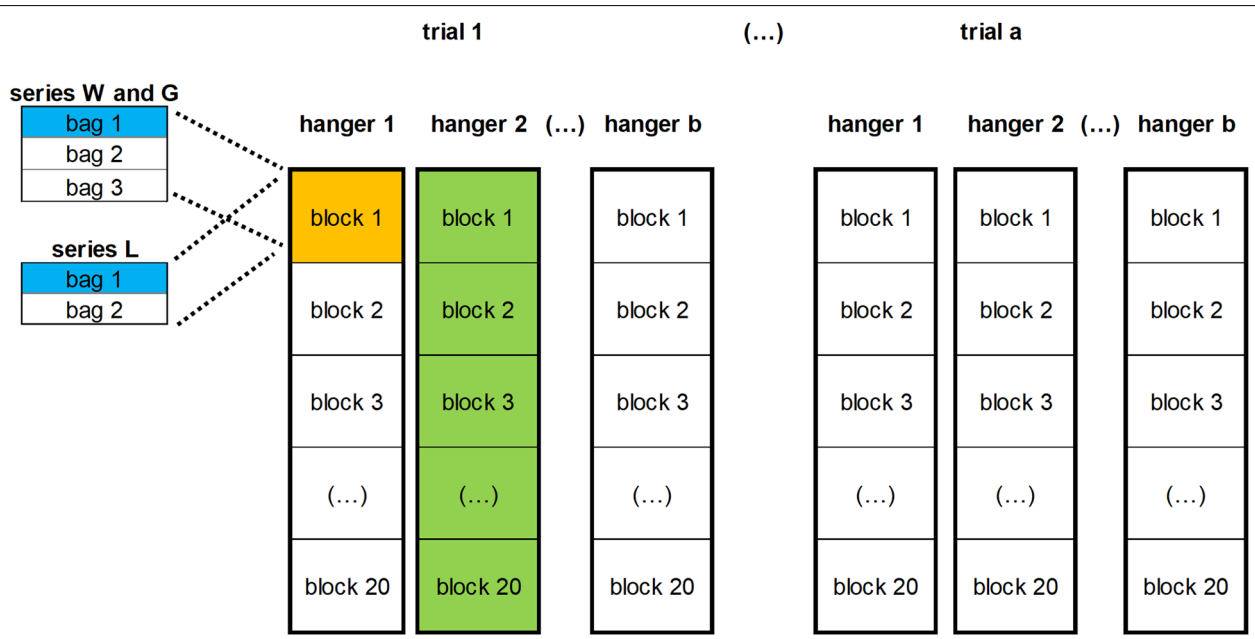


**Fig. 2** Schematic design of one series. One series comprised *a* trials (time repetitions) conducted consecutively (series W: $a=4$; series G: $a=8$; series L: $a=6$). Each trial comprised *b* hangers (hanger 2 in green) (W: $b=3$; G: $b=4$; L: $b=5$) for each *b* test factor levels. One hanger was laid out according to a randomized complete block design with 20 blocks (orange). One block comprised *c* bags (blue) (W and G: $c=3$; L: $c=2$) for each *dose* factor levels. A bag with 16 seeds was the randomization unit

### Statistical analysis

A total of 3840 bags (series W: 720; G: 1920; L: 1200) with 61,440 seeds were prepared. Trial series W, G and L were analysed separately. Growth traits are hypocotyl length from day 3 to day 6 and root length from day 2 to day 7 (Series W and G), or day 8 (Series L). These traits were independently analysed with the following mixed model by considering the trial effect within a series as random [22]:

$$Y_{ijkl} = \mu + b_i + f_j + \mathrm{bf}_{ij} + t_k + \mathrm{bt}_{ik} + \mathrm{ft}_{jk} + \mathrm{ftw}_{jkl} + e_{ijkl}, \quad (1)$$

where $\mu$ is the overall effect (intercept), $\mathrm{b_i}$ is the fixed effect of the ith *dose* treatment (Series W and G: $i=1$–3; L: $i=1$–2), $f_j$ is the fixed effect of the jth level of the test factor (W: $j=1$–3; G: $j=1$–4; L: $j=1$–5), $\mathrm{bf}_{ij}$ is the fixed effect of the interaction between the $i$th *dose* treatment and the $j$th level of the test factor, $t_k$ is the random effect of the $k$th trial (W: $k=1$–4; G: $k=1$–8; L: $k=1$–6), $\mathrm{bt}_{ik}$ is the random effect of the interaction between the $k$th trial and the $i$th *dose* treatment, $\mathrm{ft}_{jk}$ is the random effect of the $jk$th hanger, $\mathrm{ftw}_{jkl}$ is the random effect of the $l$th block in the $jk$th hanger (l = 1–20), $e_{ijkl}$ is the random effect of the $ijkl$th bag, and $Y_{ijkl}$ is the mean of root or hypocotyl length in the $ijkl$th bag.

Furthermore, three supplemental models were fitted in order to investigate other aspects of the results. They are described in Additional file 1:

- Model 2: instead of analysing the growth traits separately, hypocotyl or root growth were analysed as a whole by integrating the factor day in the model.
- Model 3: To analyse the influence of seedling position in one bag, the observation unit was defined as one seedling instead of one bag.
- Model 4: instead of analysing the whole series, each hanger was analysed separately in order to assess the reproducibility of the bioassay.

Analyses on the growth traits were performed with the MIXED procedure of the software SAS (Version 3.5, SAS Institute Inc., Cary, NC, USA). The Kenward–Roger method was used to determine the denominator degrees of freedom (option ddfm = kenwardroger) and adjust standard errors. Normality of the residual errors and variance homogeneity were checked visually. If necessary, variance heterogeneity was taken into consideration by fitting specific variances for the treatments [23].

Treatment means (main effects and interactions) were calculated and compared with the LSMEANS statement, using the Tukey–Kramer test for all pairwise comparisons to control the family-wise Type I error rate. This is a conservative approach because it also accounts for comparisons of treatments differing in both *dose* and *volume*. Another, more liberal comparison approach, which compares *doses* only at the same level of *volume*, and vice versa, is described in Additional file 1.

The influence of the treatment factor on the number of discarded seeds was investigated with Model (1), assuming a generalized linear mixed model with a binomial distribution and a logit link [24]. The GLIMMIX procedure was used with the same verifications on approximate normality and variance homogeneity of the studentized residuals as described above.

## Results

### Overall analyses over the growth period

In each series, root and hypocotyl growth were statistically analysed as a whole with Model (2) as described in Additional file 1. Table 2 presents the *p*-values of these

**Table 2 *p*-values of the fixed factors of the overall analyses in series W, G and L**

| Factor | Series W | | Series G | | Series L | |
|---|---|---|---|---|---|---|
| | Hypocotyl | Root[1] | Hypocotyl | Root[1] | Hypocotyl | Root[1] |
| Test factor[2] | < **0.0001** | < **0.0001** | < **0.0001** | < **0.0001** | < **0.0001** | < **0.0001** |
| Dose | 0.64 | 0.98 | 0.46 | **0.04** | 0.31 | **0.04** |
| Day | < **0.0001** | < **0.0001** | < **0.0001** | < **0.0001** | < **0.0001** | < **0.0001** |
| Test factor[2] × dose | < **0.0001** | **0.048** | < **0.0001** | < **0.0001** | 0.17 | < **0.0001** |
| Test factor[2] × day | **0.0005** | **0.002** | 0.93 | **0.0003** | 0.90 | 0.12 |
| Dose × day | 0.24 | **0.002** | 0.68 | < **0.0001** | 0.50 | < **0.0001** |
| Test factor[2] × dose × day | 0.96 | 0.27 | 0.91 | 0.39 | 0.73 | 0.48 |

For each series, the *p*-values (Wald *F*-test) of the analyses after Model (2) of the factors *dose*, *test factor* and *day* with their interaction are shown. Significant results are printed in bold ($p < 0.05$)

[1] Logarithmic transformation

[2] Series W: water volume; Series G: laying time; Series L: light

analyses. The main effects of *test factor* and *day* were very highly significant in every series. The main effect of *dose* factor was significant in Series L and G for root growth ($p=0.04$). In opposition, *dose* × *test factor* interactions were significant in every series, except in Series L (hypocotyl). Furthermore, in every series, *dose* × *day* interactions were very highly significant for root growth, but not significant for hypocotyl growth.

Therefore, these significant interactions involving the *dose* factor indicated a complex bioactivity of the HMP, depending on the plant growth phase and the investigated environmental co-factor.

In the following, every trait of each series was independently analysed with Model (1). To simplify the presentation of *dose* × *test factor* interactions, *test factor* levels were compared by the same *dose* treatment Control. If no other indication, the *dose* treatments $D_{1\mu l}$ and $D_{0.1\mu l}$ were compared to Control.

#### Influence of water volume (Series W)

In series W, the interaction between water volume and HMP treatment was investigated. The 4 ml treatment was prematurely stopped at day 6 because of the speed of root growth. The results are presented in Table 3 (see also Additional file 1: Table S1 for more liberal pairwise comparisons) and Fig. 3 (root growth).

Hypocotyl growth was increased by high water volume (day 6, *C* variant, 6 ml vs. 4 ml: +7.6%, 5 ml vs. 4 ml: +3.7%, $p<0.001$). No HMP effect was significant, except for a slight opposite effect by 6 ml (at day 5, $D_{1\mu l}$: −1.7%, $p=0.04$).

In contrast, root growth was reduced by high volume (day 6, 6 ml vs. 4 ml: −24.2%, 5 ml vs. 4 ml: −17.8%, $p<0.0001$). $D_{1\mu l}$ and $D_{0.1\mu l}$ tended to increase root growth after day 5 as shown in Fig. 3b. This effect was significant at day 7 for variant 5 ml ($D_{1\mu l}$: +4.3%, $p=0.048$). Hence, HMP treatment appeared to compensate the plant reactions to water overdose.

#### Effect of gravistimulation (Series G)

In series G, the interaction between the HMP treatment and the plant reactions to a gravitropic stress was investigated (Fig. 4, Table 4 and Additional file 1: Table S2).

Concerning the hypocotyl growth, significant *laying time* effects were only detected between variants 20 min and 40 min. No *dose* effect was significant.

For the root growth, laying times over 20 min significantly reduced growth compared to 1 min (at day

**Table 3 *p*-values of the fixed factors and average lengths for all growth traits in series W**

| Factor | Num D | | Hypocotyl length | | | | Root length | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Day 3 | Day 4 | Day 5 | Day 6 | Day 2 | Day 3 | Day 4 | Day 5[1] | Day 6[1] | Day 7[1, 2] |
| (a) *p*-value | | | | | | | | | | | | |
| Dose | 2 | | 0.48 | 0.28 | 0.59 | 0.55 | 0.91 | 0.38 | 0.09 | 0.36 | 0.28 | **0.006** |
| Volume | 2 | | 0.14 | 0.76 | **0.008** | **0.0004** | 0.13 | **0.004** | **0.0004** | **0.0008** | **0.001** | **0.01** |
| Volume × dose | 4 | | **0.04** | **0.007** | **0.006** | **0.02** | 0.48 | 0.25 | 0.66 | 0.68 | **0.04** | **0.0005** |
| | **Volume** | **Dose** | | | | | | | | | | |
| (b) Average length (mm) | | | | | | | | | | | | |
| | 4 ml | $D_{0.1\mu l}$ | 17.48 ab | 35.93 ab | 49.67 c | 59.86 e | 14.77 | 39.28 a | 67.35 a | 83.40 a | 91.93 a | (91.93)[2] a |
| | | $D_{1\mu l}$ | 17.45 ab | 35.91 ab | 49.90 c | 60.30 de | 14.77 | 39.21 a | 67.48 a | 83.97 a | 92.80 a | (92.80)[2] a |
| | | *C* | 17.30 b | 35.84 ab | 49.60 c | 59.83 e | 14.66 | 38.90 a | 67.88 a | 84.96 a | 93.52 a | (93.52)[2] a |
| | 5 ml | $D_{0.1\mu l}$ | 17.49 ab | 35.62 ab | 50.70 bc | 61.96 cd | 14.02 | 35.86 b | 58.07 bc | 70.13 b | 79.02 b | 84.83 ab |
| | | $D_{1\mu l}$ | 17.30 ab | 35.80 ab | 51.07 abc | 62.44 bc | 13.92 | 35.34 b | 57.06 bcd | 69.26 b | 78.92 b | **85.04** ac |
| | | *C* | 17.34 ab | 35.77 ab | 50.88 bc | 62.02 c | 13.93 | 36.00 b | 58.43 b | 69.87 b | 76.84 b | **81.34** b |
| | 6 ml | $D_{0.1\mu l}$ | 17.83 ab | 36.17 ab | 52.22 ab | 63.96 ab | 14.05 | 34.57 b | 52.49 cd | 63.48 b | 71.78 b | 77.69 bc |
| | | $D_{1\mu l}$ | 17.68 ab | **35.72** b | **51.85** b | 63.79 ab | 14.04 | 34.34 b | 51.82 d | 63.13 b | 72.67 b | 79.84 bc |
| | | *C* | 18.20 a | **36.81** a | **52.74** a | 64.35 a | 14.22 | 35.04 b | 53.59 bcd | 63.96 b | 70.92 b | 76.37 bc |
| s.e. | | | 0.45 | 1.33 | 1.24 | 1.20 | 0.54 | 1.62 | 2.19 | 2.60–2.67 | 2.81–2.94 | 2.79–2.95 |

a) For each trait, the *p*-values (Wald *F*-test) of the analyses after model (1) of the factors *dose* and *volume* and their interaction are shown. Significant results are printed in bold ($p<0.05$). b) Means (mm) and standard errors (s.e.; by variance heterogeneity: minimum and maximum) are detailed. Treatments at a time point (in column) with no letters in common differ significantly ($p<0.05$, Tukey–Kramer test). Significant differences between *dose* treatments at same *volume* level are shown in bold

*Num DF* numerator degrees of freedom

[1] Variance heterogeneity was considered for the factor *volume*

[2] For variant 4 ml, root length at day 7 was not measured. Root length at day 6 was used to perform the statistical analysis at the end of the experiment

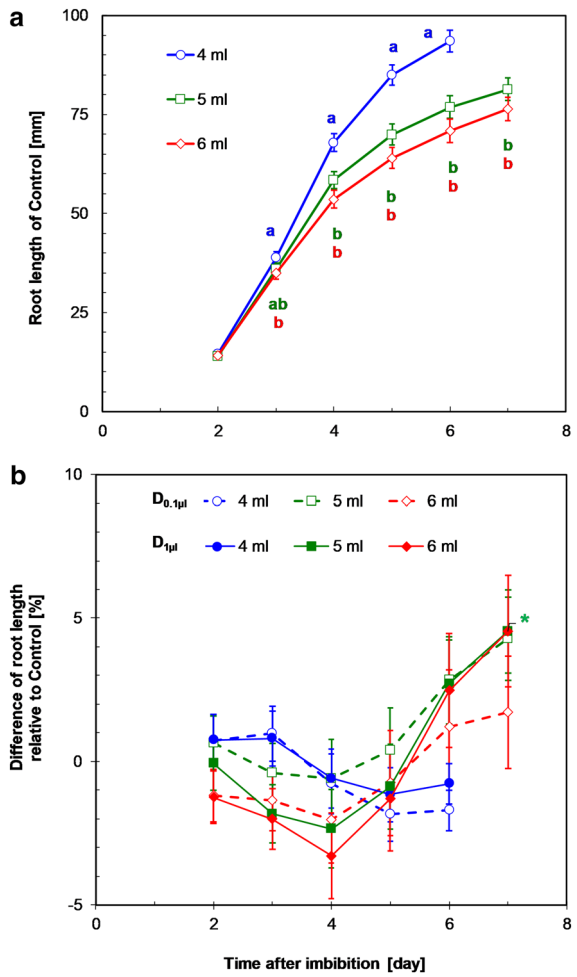


**Fig. 3** Root growth of cress seedlings in dependence with **a** *water volume* and **b** HMP *dose*. **a** One point represents the average root length for the *dose*-variant Control measured in 80 bags from 4 trials (in mm). Error bars represent ± standard error. Treatments at a time point with no letters in common differ significantly by a Tukey–Kramer test ($p < 0.001$). **b** One point represents the difference of average root length of $D_{1\mu l}$ or $D_{0.1\mu l}$ relative to average root length of Control at constant *volume* (in %, relative to control). Error bars represent ± standard error of difference. For each time point, asterisks indicate significant differences as determined by a Tukey–Kramer test: *($0.01 < p < 0.05$), **($0.001 < p < 0.01$) and ***($p < 0.001$). At day 7, the statistical analysis included the data of root length at day 6 from variant 4 ml

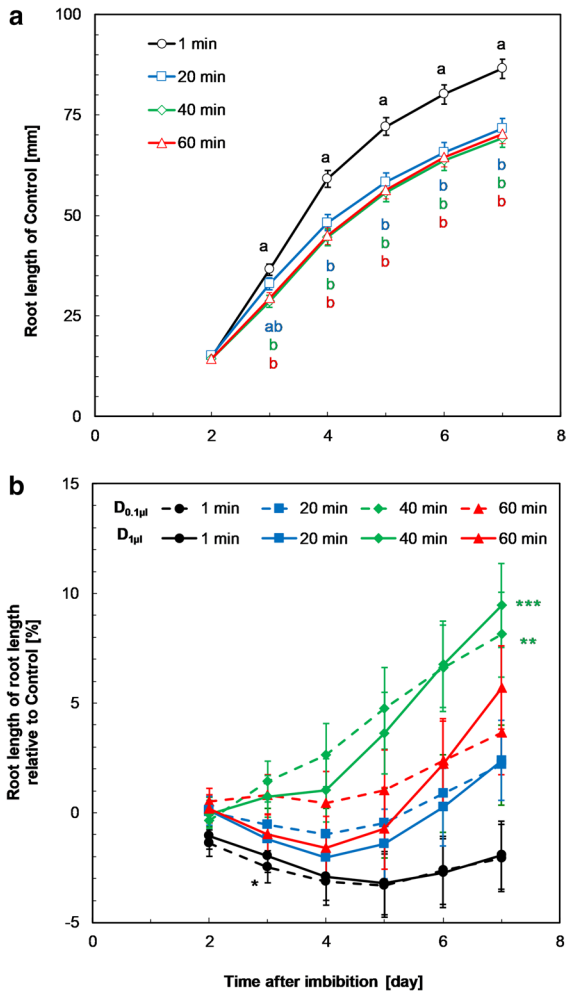


**Fig. 4** Root growth of cress seedlings in dependence with **a** *laying time* and **b** HMP *dose*. **a** One point represents the average root length for the *dose*-variant Control measured in 160 bags from 8 trials (in mm). Error bars represent ± standard error. Treatments at a time point with no letters in common differ significantly by a Tukey–Kramer test ($p < 0.0001$). **b** One point represents the difference of average root length of $D_{1\mu l}$ or $D_{0.1\mu l}$ relative to average root length of Control at constant *laying time* (in %, relative to control). Error bars represent ± the standard error of a difference. For each time point, asterisks indicate significant differences as determined by a Tukey–Kramer test: *($0.01 < p < 0.05$), **($0.001 < p < 0.01$) and ***($p < 0.001$)

7, variant *C*, 20 min: −17.1%; 40 min: −19.9%; 60 min: −18.8%, $p < 0.0001$). Under those stress conditions, the HMP treatments tended to increase root growth (Fig. 4b). This effect was significant by 40 min at day 7 ($D_{1\mu l}$: +9.5%, $p = 0.0004$; $D_{0.1\mu l}$: +8.1%, $p = 0.006$). In the contrary, by 1 min laying time, HMP treatments tended to reduce root growth (day 3, $D_{0.1\mu l}$: −2.5%, $p = 0.04$). Hence, HMP treatments appeared to compensate the plant reactions to gravitropic stress.

### Effect of light (series L)

In series L, the bags were exposed daily to natural or fluorescent light to investigate the interaction between light and HMP treatment (Fig. 5, Table 5 and Additional file 1: Table S3).

Concerning the hypocotyl growth, neither *light* effect (except at day 3) nor *dose* effect was significant.

In contrast, all FL variants tended to increase root growth compared to NL (Fig. 5a), although this effect was significant only for FL 100 (at day 8, variant *C*, FL 100 vs.

**Table 4 *p*-values of the fixed factors and average lengths for all growth traits in series G**

| Factor | Num DF | | Hypocotyl length | | | | Root length | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Day 3 | Day 4 | Day 5 | Day 6 | Day 2 | Day 3 | Day 4 | day 5 | Day 6 | Day 7 |
| a) *p*-value | | | | | | | | | | | | |
| Dose | 2 | | 0.25 | 0.31 | 0.59 | 0.24 | 0.60 | 0.09 | 0.18 | 0.70 | 0.35 | **0.02** |
| Laying time | 3 | | **0.003** | **0.01** | **0.008** | **0.008** | 0.10 | **0.0002** | < **0.0001** | < **0.0001** | < **0.0001** | < **0.0001** |
| L. time × dose | 6 | | 0.15 | **0.01** | **0.0008** | **0.008** | 0.53 | **0.02** | **0.05** | **0.02** | **0.006** | **0.0004** |
| | **Laying time** | **Dose** | | | | | | | | | | |
| b) Average length (mm) | | | | | | | | | | | | |
| | 0 min | $D_{0.1\mu l}$ | 17.37 abc | 34.72 abcd | 51.38 bcd | 62.69 cd | 14.58 | **35.62** b | 57.25 a | 69.72 a | 78.02 a | 84.75 a |
| | | $D_{1\mu l}$ | 17.54 abc | 34.99 abcd | 51.80 abcd | 63.04 abcd | 14.63 | 35.81 ab | 57.38 a | 69.80 a | 77.91 a | 84.86 a |
| | | *C* | 17.69 abc | 35.30 abcd | 51.90 abcd | 63.10 abcd | 14.79 | **36.52** a | 59.10 a | 72.12 a | 80.12 a | 86.53 a |
| | 20 min | $D_{0.1\mu l}$ | 19.22 a | 37.89 ab | 54.48 ab | 65.34 ab | 15.06 | 32.81 abc | 47.77 b | 58.03 b | 66.29 b | 73.29 bc |
| | | $D_{1\mu l}$ | 19.10 ab | 37.66 abc | 54.31 abc | 65.16 abc | 15.07 | 32.59 abc | 47.25 b | 57.48 b | 65.88 b | 73.44 bc |
| | | *C* | 19.33 a | 38.08 a | 54.68 a | 65.41 a | 15.06 | 32.99 abc | 48.24 b | 58.31 b | 65.71 b | 71.73 bc |
| | 40 min | $D_{0.1\mu l}$ | 16.14 c | 33.97 cd | 51.57 abcd | 63.31 abcd | 14.18 | 29.08 c | 45.79 b | 58.30 b | 67.77 b | **74.94** b |
| | | $D_{1\mu l}$ | 16.10 c | 33.84 cd | 51.42 bcd | 63.32 abcd | 14.22 | 28.88 c | 45.07 b | 57.67 b | 67.87 b | **75.87** b |
| | | *C* | 15.99 c | 33.50 d | 50.96 d | 62.89 bcd | 14.23 | 28.67 c | 44.62 b | 55.65 b | 63.58 b | **69.32** c |
| | 60 min | $D_{0.1\mu l}$ | 16.56 bc | 34.22 abcd | 51.21 cd | 62.39 d | 14.48 | 29.61 c | 45.12 b | 56.98 b | 65.95 b | 72.80 bc |
| | | $D_{1\mu l}$ | 16.40 c | 34.10 bcd | 51.42 bcd | 62.85 bcd | 14.43 | 29.08 c | 44.20 b | 55.99 b | 65.86 b | 74.26 bc |
| | | *C* | 16.62 bc | 34.41 abcd | 51.63 abcd | 62.90 abcd | 14.41 | 29.37 c | 44.92 b | 56.39 b | 64.42 b | 70.25 bc |
| s.e. | | | 0.71 | 1.08 | 0.78 | 0.58 | 0.28 | 1.21 | 1.76 | 1.92 | 1.87 | 1.80 |

a) For each trait, the *p*-values (Wald *F*-test) of the analyses after model (1) of the factors *dose* and *laying time* and their interaction are shown. Significant results are printed in bold ($p < 0.05$). b) Means (mm) and standard errors (s.e.) are detailed. Treatments at a time point (in column) with no letters in common differ significantly ($p < 0.05$, Tukey–Kramer test). Significant differences between *Dose* treatments at same *laying time* level are shown in bold

*Num DF* numerator degrees of freedom

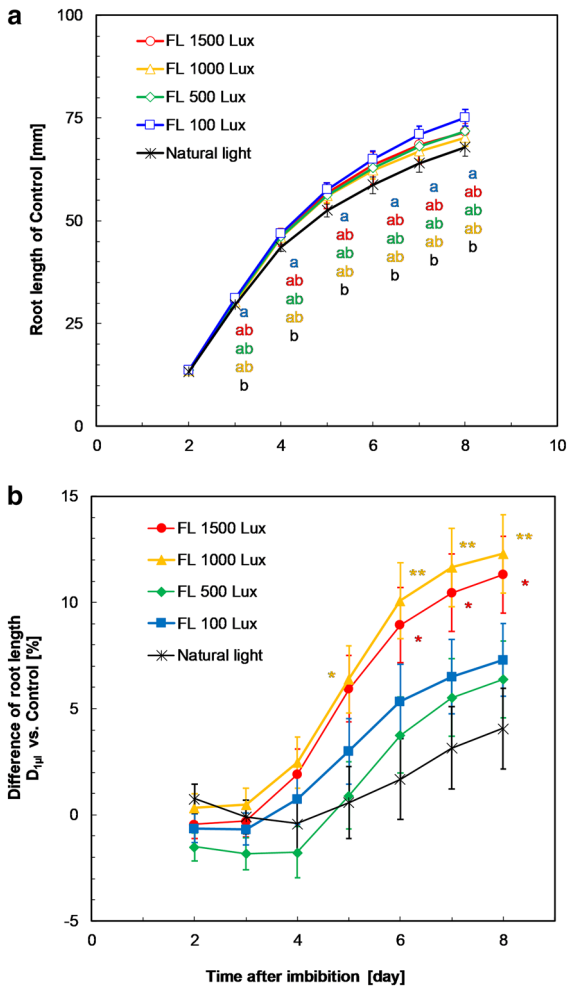


**Fig. 5** Root growth of cress seedlings with dependence on **a** *light* and **b** HMP *dose*. **a** One point represents the average root length for the *dose*-variant Control measured in 120 bags from 6 trials (in mm). Error bars represent ± standard error. Treatments at a time point with no letters in common differ significantly by a Tukey–Kramer test ($p < 0.05$). **b** One point represents the difference of average root length of $D_{1\mu l}$ relative to average root length of Control at constant *light* (in %, relative to control). Error bars represent ± the standard error of difference. For each time point, asterisks indicated significant differences as determined by a Tukey–Kramer test: *($0.01 < p < 0.05$), **($0.001 < p < 0.01$) and ***($p < 0.001$)

NL: +10.7%, $p = 0.02$). Concerning the *dose* factor, $D_{1\mu l}$ tended to increase root growth after day 4 by all *light* levels (Fig. 5b). FL at high illuminances enhanced this effect. At day 8, this effect amounted to +11.3% ($p = 0.02$) for FL 1500 and +12.3% ($p = 0.007$) for FL 1000. Hence, FL and HMP treatments appeared to interact synergistically.

### Influence of the position of the seedling in the bag

The application of the HMP suspension as one drop at the early stage of seed imbibition mimicked biodynamic practice. However, it induced a non-uniform dispersion of the HMP suspension in one bag. To investigate this concentration gradient, the interaction between the seedling position and the *dose* factor was analysed with Model 3 (Additional file 1). The examination of the interaction effects did not reveal a regular influence of the position on the treatment (Additional file 1: Figures S1–S3 for Series W, G and L, respectively). Exceptions are the variants FL 1000 Lux and 1500 Lux of Series L, where a higher HMP effect in the middle of the bag was indicated (Additional file 1: Figure S3A, B).

### Influence of the trial factor and reproducibility of the bioassay

The estimated variance parameters of the analyses with Model 1 are documented in Additional file 1: Table S4. The influence of the *trial* factor differed in the three series, as the variance of the main factor was at highest in Series W, whereas the variance of the interaction between *trial* and *dose* was important in Series L.

The reproducibility of the bioassay was investigated by analysing separately each hanger with the Model 4 described in Additional file 1. Results are presented in Additional file 1: Table S5. Significant effects of HMP treatments were detected most frequently for variant 5 ml in Series W (50% of the trials), for variant 40 min in Series G (37%), and for variants FL 1000 and FL 1500 in Series L (67%).

### Discarded seeds and bags

In all series, 4 of 3840 bags (0.1%) and 5310 seedlings from the remaining bags (8.6%) were discarded (Additional file 1: Table S6). Neither *dose* factor nor *test* factor affected significantly the number of excluded seedlings (Additional file 1: Table S7). An exception is the factor *laying time* in Series G as significantly more seedlings were discarded by 20 min compared to 40 min (+19.8%, $p = 0.01$).

## Discussion

### Interactions between the test factors and the biodynamic treatments

In Series W and G, increase of laying time and water volume significantly reduced root growth. These plant reactions were presumably due to stress caused by gravistimulation and water overdose that adversely affected the availability of oxygen to the roots [9, 25]. In those stress conditions, the application of HMP increased root growth (Series W: up to +4.3%, $p = 0.048$; Series G: up to +9.5%, $p = 0.0004$). In Series L, under combined stress

**Table 5 *p*-values of the fixed factors and average lengths for all growth traits in series L**

| Factor | Num DF | | Hypocotyl length | | | | Root length | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Day 3 | Day 4 | Day 5 | Day 6 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 | Day 8 |
| a) *p*-value | | | | | | | | | | | | | |
| Dose | 1 | | 0.45 | 0.39 | 0.20 | 0.25 | 0.34 | 0.17 | 0.34 | **0.05** | **0.03** | **0.03** | **0.03** |
| Light | 4 | | **0.008** | 0.07 | 0.16 | 0.14 | 0.27 | **0.02** | **0.009** | **0.001** | **0.001** | **0.003** | **0.004** |
| Light × dose | 4 | | 0.64 | 0.47 | 0.71 | 0.18 | 0.18 | 0.30 | 0.08 | **0.02** | **0.003** | **0.003** | **0.004** |
| | **Light** | **Dose** | | | | | | | | | | | |
| b) Average length (mm) | | | | | | | | | | | | | |
| | FL 100 | $D_{1\mu l}$ | 17.11 ab | 34.11 ab | 51.94 | 63.16 | 13.48 | 30.91 a | 47.13 a | 59.38 ab | 68.37 abc | 75.59 ab | 80.71 ab |
| | | *C* | 17.12 ab | 33.96 ab | 51.73 | 63.06 | 13.56 | 31.12 a | 46.78 a | 57.66 abc | 64.90 abce | 70.96 abc | 75.03 abc |
| | FL 500 | $D_{1\mu l}$ | 16.52 ab | 33.75 ab | 51.31 | 62.88 | 13.39 | 30.11 ab | 45.21 ab | 56.68 abcd | 65.20 abcd | 71.73 abcd | 76.24 abcd |
| | | *C* | 16.74 ab | 33.98 ab | 51.34 | 62.61 | 13.59 | 30.67 ab | 46.02 ab | 56.17 abcd | 62.84 abcd | 67.98 abcd | 71.67 abcd |
| | FL 1000 | $D_{1\mu l}$ | 16.19 ab | 33.84 ab | 51.24 | 62.29 | 13.41 | 30.29 ab | 46.93 a | **59.52** b | **68.48** ab | **74.77** ab | **78.82** ab |
| | | *C* | 16.22 ab | 33.72 ab | 51.17 | 62.23 | 13.36 | 30.14 ab | 45.80 ab | **55.95** acd | **62.20** cd | **66.97** cd | **70.19** cd |
| | FL 1500 | $D_{1\mu l}$ | 16.13 b | 33.34 ab | 51.35 | 62.50 | 13.20 | 30.39 ab | 47.02 a | 60.18 ab | **69.33** a | **75.56** b | **79.72** b |
| | | *C* | 16.15 ab | 33.09 b | 51.00 | 62.14 | 13.26 | 30.48 ab | 46.15 ab | 56.81 abcd | **63.64** bcd | **68.41** acd | **71.62** acd |
| | NL | $D_{1\mu l}$ | 17.17 a | 35.08 a | 52.17 | 62.83 | 13.34 | 29.46 b | 43.29 b | 52.74 cd | 59.66 de | 66.03 cd | 70.55 cd |
| | | *C* | 17.11 ab | 35.00 a | 52.16 | 63.12 | 13.24 | 29.49 b | 43.47 b | 52.43 d | 58.67 d | 64.01 d | 67.79 d |
| s.e. | | | 0.39 | 0.89 | 1.40 | 1.53 | 0.46 | 0.32 | 0.85 | 1.60 | 2.14 | 2.29 | 2.26 |

a) For each trait, the *p*-values (Wald *F*-test) of the analyses after model (1) of the factors *dose* and *light* and their interaction are shown. Significant results are printed in bold ($p < 0.05$). b) Means (mm) and standard errors (s.e.) are detailed. Treatments at a time point (in column) with no letters in common differ significantly ($p < 0.05$, Tukey–Kramer test). Significant differences between the *Dose* treatments at same *light* level are shown in bold

*Num DF* numerator degrees of freedom

conditions, application of fluorescent light appeared to enhance this HMP effect (up to + 12.3%, $p = 0.007$).

Therefore, these results suggested a compensatory effect of HMP by water overdose and gravitropic stress, and a synergetic interaction between fluorescent light and HMP bioactivity. The hypotheses 1, 2 and 3, respectively, were assessed. Such a stabilizing pattern of action was coherent with former results [19].

Noticeably, the HMP effect was generally independent of the seedling position, in accordance to former results [19]. Exceptions are the variants with high FL illuminance in Series L (FL 1000 Lux and FL 1500 Lux). Further research is necessary to clarify if the interactions in these variants were due to the HMP concentration gradient or to a light gradient.

### Adjusting test factors in a multi-factorial approach

The plant response to the HMP application was maximal in FL 1000 Lux of Series L (+ 12.3%), i.e. with a combination of two stress factors (water volume at 6 ml, laying time 40 min) and a synergetic interaction with fluorescent light (illuminance of 1000 Lux). The bioassay reproducibility was maximal by FL 1000 Lux as well. In contrast, no effect of the HMP application was detected if no second stress factor was applied (the case by 4 ml in Series W and by 1 min in Series G).

The stress effects on root growth remained moderate (down to − 24.2% and − 19.9% in Series W and G, respectively). No acute damage on plants was observed. Thus, life functions of plants were likely not overstrained, the observed effects presumably being due to resistance responses.

Hence, the performed multi-factorial approach appears to have successfully increased the bioassay sensitivity by keeping the overall stress levels below the threshold of a lasting damage of plants.

The HMP effectiveness affected essentially the root growth. This highlights the non-intrusive observation of root growth as a major asset of the bioassay. Furthermore, the high number of replications allowed assessing relatively small effects. Hence, this bioassay appeared to be particularly suitable to detect such small effects of biostimulants on the root system.

### Plant responses to water, light and gravity conditions

Some studies investigated the complex interactions for cress root growth specifically. Kutschera and Briggs [26] reported that geotropic responses in a population of cress seedlings were not uniform: about 52–57% of the individuals displayed a negative, 29–32% no, and 12–19% positive gravitropic root responses. This inhomogeneity could partly explains the response variations

described in the present results. Furthermore, the interactions between phototropism and gravitropism were thoroughly investigated by Hart and MacDonald [27, 28]. They established that the sensitivities of etiolated and green seedlings differed. For etiolated seedlings (the case in the present study), the gravitropic responses depended on the extent of previous exposure to light. The authors reported effects of short exposures to light (5 min red or blue light) on geocurvature of hypocotyls. Moreover, light effectiveness was enhanced if the geotropic stimulus was simultaneously applied. Noticeably, this was the case in the present study, although the light exposure was shorter (1 min, but repeated daily). In overall, the present results confirmed the high sensitivity of the cress roots to light and geotropic stimuli and the interactions between them.

Typically, plant sensitivities to water, gravity and light induce major sensory systems responsible for regulating plant growth. Their interactions are fundamental to understand root growth, as each stimulus can enhance or reduce the effectiveness of the other. Interactions between gravitropism and phototropism may be partly due to the common elements in the signal transmission of both sensory systems: principally auxin, but also $Ca^{2+}$ and ethylene [26, 29]. Indeed, according to the well-established Cholodny–Went theory, auxin is the signal for gravitropism and phototropism as the auxin redistribution on opposite sides of the stimulated organ lead to the tropic growth. However, the auxin transport regulation and the perception systems for gravitropism and phototropism are thought to differ [30]. Regarding plant reactions to waterlogging, the triggered biochemical processes include ethylene synthesis and changes in auxin and cytokinin concentrations [25]. Therefore, plant reactions to the three investigated stimulations appeared to involve common elements like auxin and ethylene.

Noticeably, the root sensitivity to direct light is also of importance for the present bioassay, but this sensitivity had been mainly overlooked [31].

#### Hypothesis on the bioactivity of HMP

Despite the complexity of the three sensory systems and their interactions, one can focus on the role of auxin that is involved in gravitropism, phototropism and waterlogging. By assessing the interaction between the HMP and these environmental cues, the present results raise the question if the HMP is related with auxin. Some studies investigated this relation. Giannattasio et al. [10] reported an auxin-like activity by concentration of 1 g HMP $L^{-1}$ that was higher than in the present study. Radha and Rao [32] reported the presence of auxin-producing bacterial strains in HMP. However, Botelho et al. [33] did not detect indole-3-acetic acid (IAA) in HMP.

However, investigations on HS showed that the auxin-like activity is not always correlated with the presence of auxin. Auxin-like activity of HS had been corroborated by the stimulating inducing effect of HS on lateral root formation. Trevisan et al. [34] established that this HS effect is induced by the transcription of the auxin responsive gene. Auxinic activity did not always correlate with the small amount of IAA detected in HS, but seems to also be connected to the presence of compounds that might stimulate the plant endogenous metabolism of auxin [5]. Indeed, effects of HS on plants are complex, involving non-linear, cross-interrelated processes, and they can display auxin-, gibberellin- and cytokinin-like activities [35, 36]. The relationships between structure and bioactivity of HS are hardly unravelled due to the lack of detailed knowledge on the composition. However, only low molecular size fractions with high content of aromatic, carboxylic and phenolic groups appeared to induce morphological changes similar to those caused by IAA [37]. Interestingly, Spaccini et al. [12] detected the presence of those molecular groups in HMP by establishing high content of aromatic lignin derivatives and carboxyl groups in aliphatic acids of plant and microbial origin. The authors suggested that HMP may be more biolabile in soil and more bioactive toward plant growth than common compost having undergone full aerobic fermentation.

Indeed, the decomposition and fermentation processes that lead to the formation of HS can influence very specifically their bioactivity [37]. For example, forest soils can be better differentiated with their bioactivity than with the soil chemical parameters [38]. This highlights the importance of the specific maturation of HMP occurring underneath in winter and in cow horns. It is characterized by its slowness, anaerobic conditions and small quantities. These conditions could explain the development of the specific bioactivity described in the present results.

## Conclusions

The objective to increase the bioassay sensitivity by adjusting the water, gravity and light conditions of plant growth was successfully met. The present results assessed the interactions between the HMP and the plant reactions to the three factors. However, the observed significant responses should be further validated in soil bioassays and in field trials to evaluate their relevance for the agricultural practice. Further bioassay refinement could aim at improving the stability by standardizing uncontrolled factors. Other factors like the temperature and the composition of the bag solution can be adjusted as well. In regard to the non-intrusive observation of root growth and the high test

power, this bioassay appeared to be particularly suitable to detect small effects on the root system. A similar multi-factorial approach can be used to adjust it to other biostimulants.

## Supplementary information

**Supplementary information** accompanies this paper at https://doi.org/10.1186/s40538-020-0176-x.

**Additional file 1.** Additional statistical models, tables and figures.

### Abbreviations

HMP: Horn manure preparation; HS: Humic substances; IAA: Indole-3-acetic acid; FL: Fluorescent light; NL: Natural light; s.e.: Standard error.

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### Authors' contributions

AM conceived and conducted the experiments. AM and HPP analysed the data statistically. AM interpreted the results and wrote the manuscript. HPP reviewed the manuscript. All authors read and approved the final manuscript.

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### Availability of data and materials

The datasets generated during the current study are available from the corresponding author on reasonable request.

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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## Publisher's Note