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Milli-channel array for direct and quick reading of root elongation bioassays

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ABSTRACT

A novel platform to perform systematic analysis and direct reading of root elongation bioassays is presented. The device was designed to include multiplexed microenvironments for the germination and growth of individual seeds, which allows observation by the naked eye or by optical systems, notably cellphone cameras. Prototypes were fabricated by laser micromachining on a highly transparent material that is fully compatible with biological systems. The effectiveness of the milli-channel array was verified against the conventional system (Petri dish). *Lactuca sativa* was chosen as a model species and glyphosate as a typical toxic agent. All tests were run according to standardized procedures and data analysis was carried out through different statistical indicators such as the root elongation and germination indexes. Results attained in the milli-channel array were identical to those in Petri dish, with the remarkable benefit that several steps required in the conventional system were avoided, which enormously decreases the operation time and the possibility of experimental errors. Further advantages of the milli-channel array are also reported, such as the capability to achieve live imaging of plant organs growth through a simple experiment. The developed device has been proven to be effective, versatile, easy-to-use, and integrable to cellphones, which naturally provide facilities for data recording, analysis, and networking. These improvements open the route to novel applications of bioassays in the wide field of ecotoxicology and environmental studies.

1. Introduction

Since the 1980s, the use of phytotoxicity tests based on plants and algae has been continuously growing because of their demonstrated value for toxicological assessment (Wang, 1991; Gopalan, 1999; Lyu et al., 2018; Chandrasekhar and Ray, 2019). Among other plants, *Lactuca sativa* is a model species for phytotoxicity assays; it has been recommended by international organizations to be used for standard toxicity tests, as well as for studying ecological effects of potentially toxic substances (ISO, 1995; US EPA, 1996; OECD, 2003; IRAM 29114, 2008). Phytotoxicity tests using *L. sativa* are simple, reliable, and cheap (Wang and Williams, 1990; Charles et al., 2011; Park et al., 2016; Lyu et al., 2018). In particular, as germination is the first step of material exchange between the developing plant and the environment, both the number of germinated seeds and the root elongation are sensible parameters for phytotoxicity testing (Wang and Williams, 1988; Wang and Keturi, 1990; Araújo et al., 2001; Di Salvatore et al., 2008; Park et al., 2016; Lyu et al., 2018). Therefore, this practical and economical method is being constantly used for environmental monitoring (Chan-Keb et al., 2018; Mtisi and Gwenzi, 2019), wastewater quality control (Aguiar et al., 2016), ecotoxicity responses (Utzig et al., 2019), and soil remediation (Rede et al., 2016; Chandrasekhar and Ray, 2019).

Bioassay toxicity of *L. sativa* seeds is a 120 h lasting assay, in which germination and root elongation take place under the exposure to static toxic species (pure compounds or complex mixtures). The assay is normally carried out in Petri dishes (PD), where 20 seeds are promoted to germinate on paper as material substrate, which is conveniently imbibed with the toxic solution. The grown roots are often mixed and entangled, hence the steps of extraction, ordering, and alignment are necessary. It is worth noting that these steps are time-consuming, highly-demanding for the operator, and consequently favor the lack of reproducibility.

Solving this problem is precisely the objective of the present work. In fact, a novel platform is proposed to implement the toxicology bioassays, which is simply designated as milli-channel array (MA). The MA (see below Fig. 1) has the following advantages: (*i*) the platform is fully transparent to allow direct observation and imaging; (*ii*) the chip contains 20 differentiated milli-channels for the germination and growth of individual seeds, thus the elongated roots can be directly measured on the platform by straightforward comparison to an integrated ruler, or (*iii*) they can be imaged by using a USB microscope or simply the cellphone camera, which also enable image analysis and data management, greatly improving the efficiency of the entire bioassay; (*iv*) the observation/imaging can be made without

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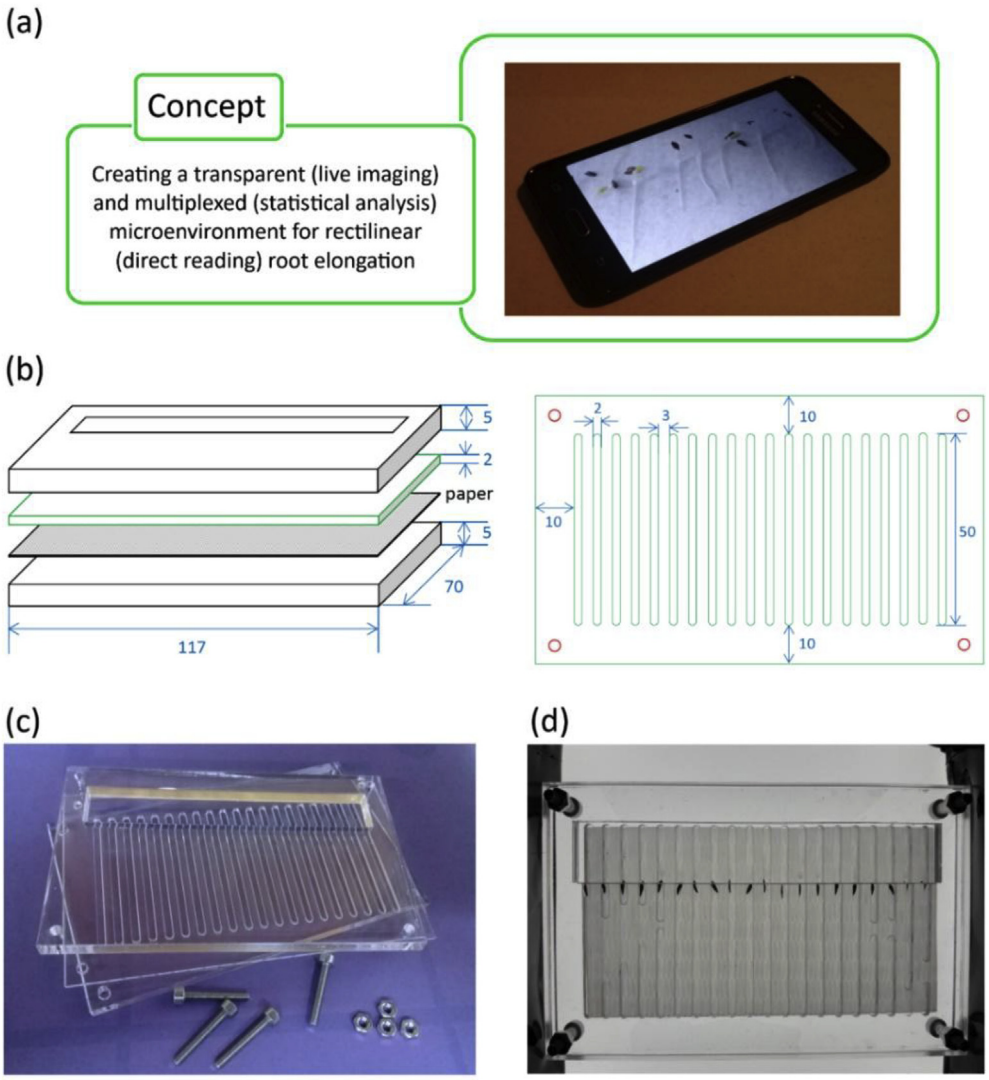

Fig. 1. (**a**) Sketch of the experimental concept that lead to design, fabrication, and testing the MA. (**b**) Design: exploded view of the multilayer system (left panel; drawings are out of scale) and details of the middle layer containing an array of 20 parallel milli-channels for root confinement (right panel); dimension are in mm. (**c**) Fabrication: PMMA layers machined by laser ablation. (**d**) Experiment: ensembled prototype including the germination paper; the picture was taken during preliminary steps of a typical test, after placing the seeds in the predefined germination site.

interrupting the experiment (instead, PD assays can be evaluated only once during the test), which enable the study of the root elongation process as a function of time, gathering rich quantitative information along the assay, and finally, (*v*) the chip can be cleaned, ensembled, and reused many times, being the paper substrate the only disposable component.

The design proposed was inspired by the microfluidic platforms for environmental organismal studies, an emerging field of research termed Soil-on-a-Chip (Stanley et al., 2016), which is closely related to the already established field of Organ-on-a-Chip (Bhatia and Ingber, 2014). Microfluidic technology provides precise control over the microenvironments together with high-resolution imaging, which leads to an unprecedented view of biological events at soil organisms, including bacteria, nematodes, fungi and plants (Aleklett et al., 2018). Thus, microfluidic platforms constitute a powerful and flexible tool to evaluate the toxicology of herbicides on chip-based bioassays that, among other advantages, include high throughput, reproducibility, and improved data statistics due to parallel processing. Prior developments in microfluidic devices have greatly advanced the analyses of model biological systems, such as *Drosophila melanogaster* (Lucchetta et al., 2005; Ghaemi et al., 2015), *Caenorhabditis elegans* (Rohde et al., 2007; Krajniak and Lu, 2010), and *Arabidopsis thaliana* (Meier et al., 2010; Park et al., 2017). Developing these microfabricated platforms is essential for plant biology research and comparative toxicological assessment (Jiang et al., 2014; Elitaş et al., 2017; Lyu et al., 2018).

The MA presented in this work was developed following the typical steps in microfluidic technology: concept, design, fabricate, and experiment, as illustrated in Fig. 1. The prototypes were made of polymethyl methacrylate (PMMA), a thermoplastic polymer that presents excellent optical properties and is fully compatible with biological systems (Hou et al., 2017). In addition, PMMA is mechanically resistant and enables laser ablation micromachining (Jensen et al., 2003; Hong et al., 2010). To verify the feasibility of the prototypes, experiments were run to evaluate the effect of glyphosate [*N*-(phosphonomethyl) glycine] on seed germination and root elongation of *L. sativa*. This organophosphorus compound is widely used as broad-spectrum herbicide, usually employed in the form of a glyphosate isopropylamine salt (Van Bruggen et al., 2018). Assays employing Zn (II) were also implemented, which is stablished in the normative as the “positive control”. The tests were carried out in parallel with PD assays to crosscheck the MA performance, which was made through different standard determinations. Furthermore, to explore novel applications, we report on

a simple experiment with great potential in the field of environmental research: the direct observation of plant development, following the temporal evolution of plant organs, and thus collecting relevant biological data. Finally, it is worth to mention that we have employed cellphone cameras for data reading and handling, as a first step to further integration and connectivity. In fact, nowadays smartphone technology is being transformative for lab-on-a-chip applications in different fields.

2. Materials and methods

2.1. Millichannel-array fabrication

Highly transparent PMMA sheets were acquired in the local market (Acrimev, Santa Fe, Argentina). A CO_2 laser platform for manufacturing applications (PLS Platform, Universal Laser System, Scottsdale, USA) was used for PMMA cutting and engravement. PMMA devices were prototyped in three rectangular layers of 70 mm × 117 mm (Fig. 1b and c). Sheets 4 mm thick were used for the base and top layers, the last one having a rectangular window of 15 mm × 100 mm. A sheet 2 mm thick was used for the middle layer, which contains 20 equal slits 2 mm width and 50 mm long (Fig. 1b, right panel). Between the base and the middle PMMA layers, a germination paper was included, which works as the substrate material for seeds germination and roots elongation. All the layers were ensembled by using stainless-steel screws. The resulting microenvironment for root growth consists in straight (50 mm), square (2 mm × 2 mm) cross-section channels, with germination paper on the bottom (Fig. 1d). The top PMMA layer has a rectangular window, thus the upper section of the milli-channels (15 mm) are open to the atmosphere, while the lower section (35 mm) is covered to ensure rectilinear root elongation. In addition, the window border serves to place the seeds in a predefined position, as it is shown in Fig. 1d.

2.2. Materials

L. sativa seeds were provided by FeCoAgro (Federación de Cooperativas Agropecuarias, Argentina). The seeds were stored at 4 °C in the dark. A commercial formulation of glyphosate (Credit®, Nufarm S.A., Argentina) containing isopropylamine salt of glyphosate at 480 gL^{-1} as active ingredient (equivalent to 48% w/v of glyphosate) was used. Six serial dilutions were prepared using distilled water in the following concentrations: 0.01, 0.1, 1, 10, 100 and 1000 mgL^{-1}. Germination paper quality 0859 (Munktell®, Sweden) was employed. The parallel PD tests were made by using disposable plastic Petri dishes.

2.3. L. sativa bioassay

The bioassays were applied according to US EPA (1996) procedures with slight modifications. A single layer of germination paper was used, the area of which was 7850 mm^2 for MA and 5000 mm^2 for PD. Both MA and PD assays employed 20 seeds of almost identical size and color. In PD assays, the seeds were laid on the germination paper with a more or less equidistant distribution. In MA assays, the seeds were gently placed into the channels, well ordered at the onset of the open window (Fig. 1d). Both assays employed 0.51 μL of the working solution per mm^2 of germination paper, meaning 4 mL for PD and 2.55 mL for MA. The devices were kept under controlled conditions of temperature (24 ± 1 °C) during 120 h. Devices were settled horizontally and a lid was placed over the chips in order to maintain the relative humidity (60–70%), which was controlled by using a digital thermohygrometer (SCHWYZ, Switzerland). A negative control test employing only distilled water was run for each system. All the assays were performed in quadruplicate (n = 80).

2.4. Data treatment

For reading, the cellphone camera was used to capture top images of the integral MA, then the images were readily treated to evaluate root elongation. Pictures recorded were analyzed by using the ImageJ© software (National Institutes of Health, USA). The germinated seeds were counted and the root length of the germinated seeds were measured. The collected data were used to determine the following toxicity indicators.

The elongation root index was calculated as,

$$RE = \frac{E_s - E_c}{E_c} \quad (1)$$

where E_s is the average root length attained for each sample concentration and E_c is the average root length attained in the negative control. The *RE* index ranges from −1 to 1 and statistically represents the normalized residual root elongation of germinated seeds for each treatment. According to Bagur-González et al. (2011), $RE < 0$ suggests that the samples are suffering toxicity. A simple scale to classify toxicity on the base of *RE* is as follows: 0 to −0.25, low; −0.25 to −0.5, moderate; −0.5 to −0.75, high; −075 to −1, very high. Conversely, $RE > 0$ indicates a stimulation of the seed growth (hormesis).

The germination index was calculated as,

$$G(\%) = \frac{N_s E_s}{N_c E_c} 100 \quad (2)$$

where N_s is the effective number of germinated seeds for each sample concentration and N_c is the effective number of germinated seeds in the negative control. The *G* index was used to assess the response variability among the different glyphosate dilutions. In previous works (Zucconi et al., 1985; Ortega et al., 2000), values of *G* under 60% are considered phytotoxic.

Statistical data treatment employed analysis of variance (ANOVA) with Duncan test a posteriori, at a level of 95% confidence using free software (R program version 2.3.3.3). Multifactorial design was proposed, where factors correspond to “glyphosate concentrations” and “systems”. In addition, an ANOVA test was used to compare slopes and intercepts of different regression curves at a level of 90% confidence.

3. Results and discussion

3.1. Evaluation of the MA performance

3.1.1. Root elongation measurements

Firstly, it is relevant to point out the overall result: the proposed MA was effective to enable appropriate seed germination and the subsequent direct observation of root elongation, meaning that both the design was suitable and the materials were properly selected. In what follows we describe the results of experiments made in parallel to PD assays, under the same conditions, with the same germination paper, and equal number of *L. sativa* seeds, in order to quantitatively evaluate the MA performance.

Fig. 2 displays the experimental outcome of each assay format: PD and MA, respectively. In addition to the toxic glyphosate (Fig. 2a and b), assays employing an inorganic salt, more precisely zinc sulphate (Fig. 2c and d), which is stablished in the normative as the positive control. It is worth to remark that, while the PD requires plants extraction, ordering, and root alignment, the MA immediately allows optical inspection and measuring. In fact, the possibility of direct reading, either by the naked eye or after image capturing, greatly simplify the results evaluation, thus decreasing both the evaluation time and the potential experimental failures.

The width of the milli-channels (2 mm; Fig. 2b and d) has been chosen as a tradeoff between two boundaries: wider channels would increase the root curvature and thinner channels could produce some confinement effect on the root elongation. Experimental results show

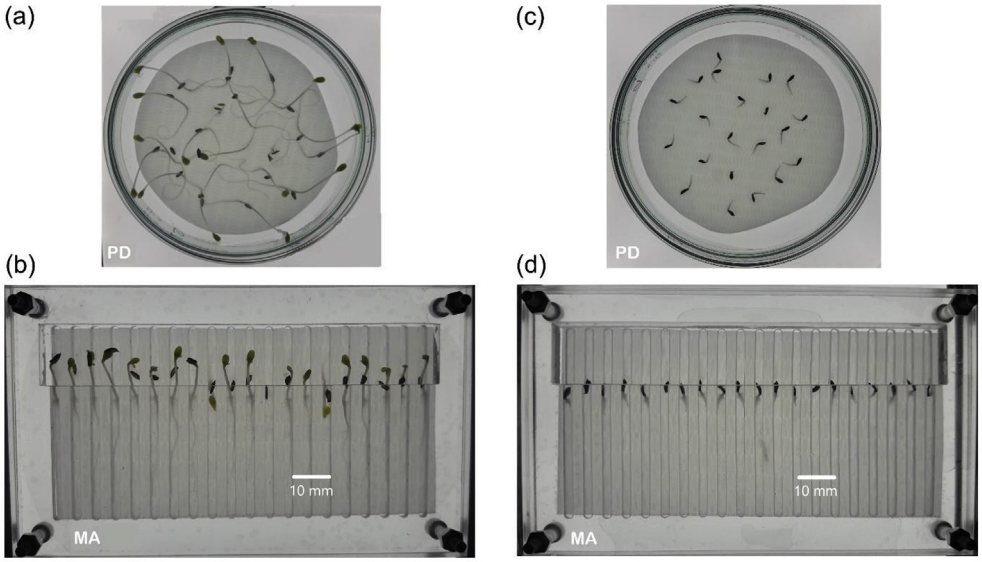

Fig. 2. Pictures of the devices at the end of the experiments: (**a**) PD and (**b**) MA, both for the minimum glyphosate concentration (0.01 mgL^{-1}); (**c**) PD and (**d**) MA, both for zinc sulphate (1000 mgL^{-1}).

Table 1
Root elongation of *L. sativa* in MA and PD experiments.

Control	System	$\bar{E}$ (mm)	SD	CV
	MA	17.4	5.2	29.7
	PD	21.8	6.4	29.6
Glyphosate (mg L^{-1})				
0.01	MA	21.9	5.3	24.1
0.01	PD	24.3	8.8	36.0
0.1	MA	19.0	7.4	39.2
0.1	PD	23.5	8.5	36.0
1	MA	15.8	6.1	38.2
1	PD	21.7	7.0	32.1
10	MA	10.3	3.3	32.4
10	PD	13.5	4.0	29.5
100	MA	4.7	0.9	19.7
100	PD	6.3	2.1	33.3
1000	MA	4.0	1.0	24.6
1000	PD	4.1	1.2	29.1
Zinc sulphate (mg L^{-1})				
1000	MA	3.1	1.0	33.1
1000	PD	2.5	0.6	23.8

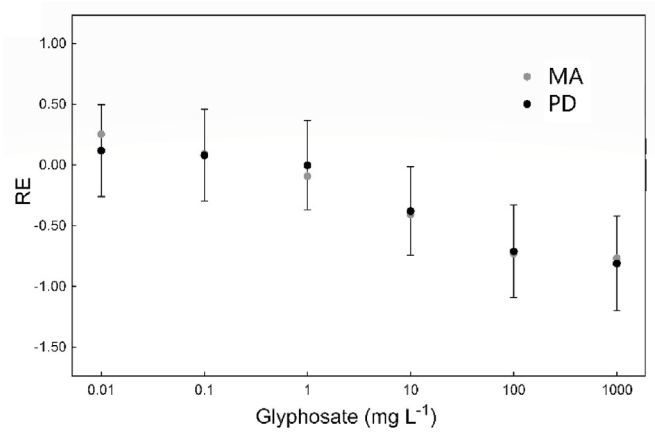

Fig. 3. Root elongation index of *L. sativa*, as a function of glyphosate concentration, for each experimental setup: MA and PD. Calculations were made according to Eq. (1) (**a**). Pictures of the devices at the end of the experiments: PD (**b**) and MA (**c**); both for the minimum toxic concentration.

that the elongation distances attained in each device (PD and MA) are perfectly comparable, meaning that the chosen width is appropriate (see Table 1 and Fig. 3 below). Consistently with these results, it is important to take into account that, in the PD method, the manually extended roots do not result perfectly straight; thus, the slight curvature induces a similar level error in both systems.

Table 1 reports measured elongation root from the whole tests, including data of a wide range of glyphosate concentration, data of the assays with zinc sulphate, and control experiments. In this table, $\bar{E}$ is the mean root elongation value, SD is the standard deviation, and CV is the coefficient of variation.

3.1.2. Root elongation (RE) index

Fig. 3 presents the *RE* index as a function of the glyphosate concentration. Data constitutes a dose-response curve, where it can be readily observed how root elongation was reduced with increasing levels of glyphosate. While the general result is expected, it is remarkable for the purposes of this work that measurements in the MA coincided with those in the PD format, for a wide range of glyphosate concentration.

In order to attain statistically meaningful indicators of the MA performance, the ANOVA test was carried out against the standard PD system, by considering “glyphosate concentrations” and “systems” as factors. As the P-value obtained for “glyphosate concentration” was less than 0.05 and P-value for “systems” was greater than 0.05 (at the 95.0% confidence level), the results shown that the first one has statistically significant effect on the response (*RE*, Fig. 3) at the 95.0% confidence level. There are five statically different homogeneous groups (0.01, 0.1, 1, 10 mgL^{-1} and (100, 1000 mgL^{-1})). All the averages for “glyphosate concentration” were different, except for the two highest values. Furthermore, the factor “systems” has no statistical effect on the response (*RE*), since there is one homogenous group (MA, PD). Therefore, the performance of the MA was successfully checked.

3.1.3. Germination (G) index, toxicity and phytotoxicity levels

An additional validation test was implemented: the evaluation of the *G* index, which takes into account the number of germinated seeds, as described above. Once more, the results attained in the MA are compared against those in PD for different glyphosate concentrations, as reported in Fig. 4. It could be observed that measurements in both experimental setups coincides for the whole range of glyphosate

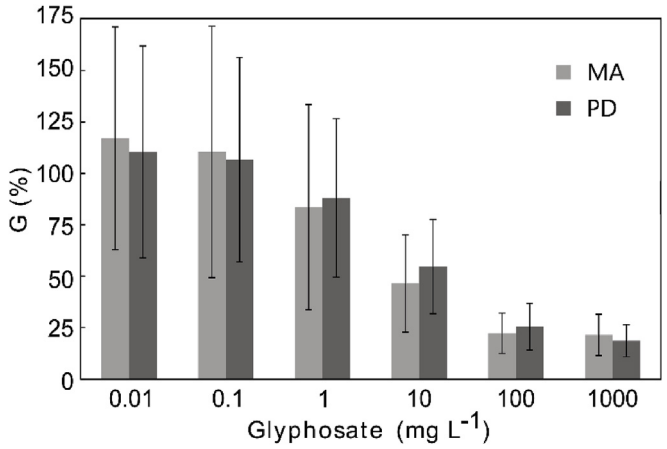

Fig. 4. Germination index as a function of glyphosate concentration obtained in each experimental setup: MA and PD. Calculations were made according to Eq. (2). It is worth noting that the control corresponds to $G = 100\%$.

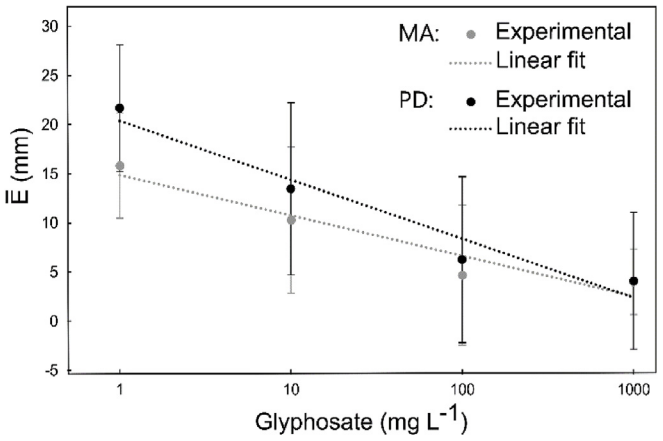

Fig. 5. Average root elongation as a function of glyphosate concentration for each experimental setup: MA and PD. Symbols are experimental data ($n = 80$) and dotted lines are the respective regression curves: Eq. (3) and related text.

concentration.

In addition, toxicity and phytotoxicity levels were compared employing both systems in order to reinforce the validation of MA. Table 2 summarizes the toxicity and phytotoxicity levels (ranges) obtained in each system (MA and PD). It can be noticed that among the glyphosate concentrations evaluated, all toxicity levels were detected (Low, Moderate, High and Very High) being the same for each glyphosate concentration for both systems. In addition, one may observe that there is a stimulation of root elongation on both systems for 0.01 and 0.1 mgL^{-1} of glyphosate ($RE > 0$, Fig. 3; $G > 100\%$, Fig. 4). Furthermore, phytotoxicity for all glyphosate concentrations was the same for both systems.

3.1.4. Mean effective concentration (EC50)

A relevant aspect in toxicology is the relation between the concentration of a toxic substance in contact with an organism (plants, animals, microorganisms, humans) and the harmful effects that produce. Thus, the dose-response relationship is important to evaluate the risk caused by chemical substances on the environment. The aim is to determine the mean effective concentration (EC50), that is, the required concentration of a toxic compound to reduce root growth on a 50% respect to a negative control. This determination is usually the first experiment performed in toxicology analysis.

Assays employing *L. sativa* seeds were used to evaluate the EC50 in both setups (MA -PD). Fig. 5 presents the average ($n = 80$) root elongation $\bar{E}$ as a function of glyphosate concentration. In this figure, the straight lines correspond to the simple regression analysis performed to determine the EC50 value for each system, by using the equation,

$$\bar{E} = a + b \ln(C_s) \quad (3)$$

where C_s is the sample concentration (glyphosate, in mgL^{-1}) and $\bar{E}$ is given in mm. The best fits to these data correspond to the following parameters: $a = 20.4$, $b = -2.6$, $R^2 = 0.947$, for PD, and $a = 14.9$, $b = -1.8$, $R^2 = 0.922$, for MA.

The performance of both systems (MA-PD) for EC50 estimation was statistically performed through the comparison between the slopes and intercepts of the regressions employing ANOVA test. P-values were 0.5789 for intercepts and 0.7378 for slopes. It can be seen that, since P-values for intercepts and slopes were > 0.1, there is not a statically meaning difference between them (at 90% confidence). These successful results demonstrate that the MA can be used as an effective method for determining the EC50.

Table 2
L. sativa bioassays results.

Glyphosate (mg L^{-1})	Level of toxicity[a]		Phytotoxicity[b]	
	MA	PD	MA	PD
0.01	> 0	> 0	–	–
0.1	> 0	> 0	–	–
1	Low	Low	–	–
10	Moderate	Moderate	Yes	Yes
100	High	High	Yes	Yes
1000	Very high	Very high	Yes	Yes

[a] Bagur-González et al. (2011).
[b] Zucconi et al. (1985); Ortega et al. (2000).

3.2. Further advantages: assessing the temporal evolution of the root length

In previous sections the effectiveness of the MA to carry out bioassays under recommended guideline was verified, showing that the novel format is an advantageous alternative to standard Petri dishes. In addition, as mentioned in Section 1, the MA was designed as a platform to allow direct observation of the plant development, without interrupting the experiment. In order to proof this concept, this section reports the results obtained by imaging the temporal evolution of the growing plant. Experiments were run under the same conditions described above, without toxic agents (only water).

Fig. 6a presents the average root length measured at different time intervals along 120 h. To gather these data, images were captured with a cellphone camera and the elongated roots were simply measured by comparison to known scales (milli-channels length). For the purposes of illustration, Fig. 6b and c displays typical captures of the MA during the experiment, where the dashed red lines indicate the average root elongation $\bar{E}$. It is clearly seen how the microenvironment of the transparent milli-channels enables the optical tracking of the germination and growth of individual seeds. This versatile platform can be further functionalized to perform more complex assays in which live imaging can provide rich information on plant development, not only for toxicants but also for growth promoting agents.

4. Conclusions

The present work proposes a novel platform to perform seed germination and root elongation assays, which has notable advantages in comparison to the standard Petri dish format. The concept of making a transparent and multiplexed microenvironment for direct reading of root elongation was fully accomplished: the prototypes were designed, fabricated, and thoroughly tested, where they have proven to be highly effective, versatile, and easy-to-use. The cost of each device is about 20

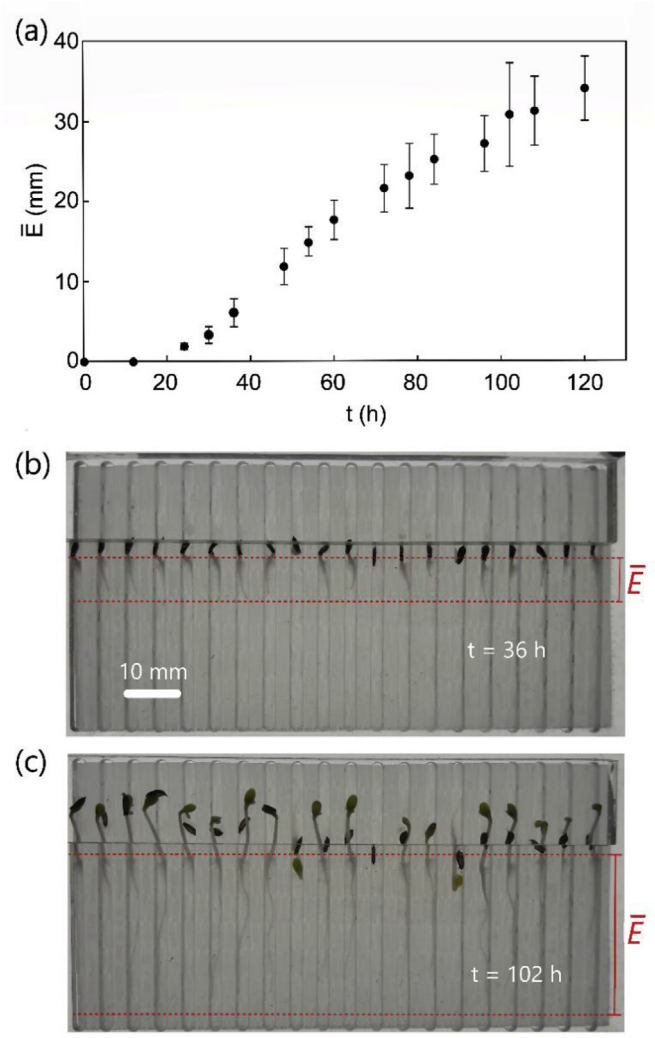

Fig. 6. Average root elongation of *L. sativa* as a function of time (**a**). Typical images of the MA showing the growing roots at different time steps: 36 h (**b**) and 102 h (**c**); the red bar indicates the average root elongation $\bar{E}$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

US dollars (as made in our lab, not mass production). However, the advantage of this technology is not the relatively low cost, but systematization, which avoids several steps of the standard method (plants extraction, ordering, root alignment), thus enormously decreasing the operation time and the possibility of experimental errors.

The milli-channel array has also been conceived to allow live imaging of the plant development; this possibility was demonstrated through a simple experiment of root elongation as a function of time. Images of the time-dependent processes can be captured with different optical facilities, then recorded and handled. Furthermore, the chip can be easily functionalized to observe other plant organs in more details, which would provide valuable information for other research fields related environmental studies. One may finally conclude that the developed platform is a clear improvement over the existing technology, with an additional advantage: the coupling to cell phones, which naturally provide facilities for data recording, analysis, real-time shearing, and networking. Therefore, novel applications of toxicity bioassays are envisioned for different fields related environmental research, from agricultural production to biobeds monitoring.

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