Checcucci *et al. Plant Methods* (2025) 21:58
https://doi.org/10.1186/s13007-025-01378-5

Plant Methods

METHODOLOGY

Open Access

Phenotype microarray-based assessment of metabolic variability in plant protoplasts

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Abstract

Background Productivity and fitness of cultivated plants are influenced by genetic heritage and environmental interactions, shaping certain phenotypes. Phenomics is the -omics methodology providing applicative approaches for the analysis of multidimensional phenotypic information, essential to understand and foresee the genetic potential of organisms relevant to agriculture. While plant phenotyping provides information at the whole organism level, cellular level phenotyping is crucial for identifying and dissecting the metabolic basis of different phenotypes and the effect of metabolic-related genetic modifications. Phenotype Microarray (PM) is a high-throughput technology developed by Biolog™ for metabolic characterization studies at cellular level, which is based on colorimetric reactions to monitor cellular respiration under different conditions. Nowadays, PM is widely used for bacteria, fungi, and mammalian cells, but a procedure for plant cells characterization has not yet been developed, due to difficulties linked in identifying a suitable reporter of cell activities.

Results Here, we tested for the first time, PM technology on plant cells using protoplasts as a means of evaluating metabolic activity. Indeed, studying the metabolism of plant protoplasts can be a valuable method for predicting the inherent metabolic potential of an entire plant organism. Protoplasts are indeed valuable tools in plant research and biotechnology because they offer a simplified, isolated cellular system where researchers can focus on intracellular processes without interference from the cell wall. As a proof-of-principle, we used protoplasts of *Solanum tuberosum L*. as model system. Protoplasts were isolated from leaf tissue of in vitro-grown plants, purified and then diluted until desired concentration. Microplates were inoculated with protoplasts suspension and various markers of redox potential as indicators of cell activity were tested. After identifying the optimal conditions for PM testing, metabolic tests were extended to protoplasts from *S. lycopersicum L.,* evaluating plant response to different NaCl concentrations and some of the toxic compounds present in pre-configured Biolog™ microplates.

Conclusions The standardized high-throughput system developed was effective for the metabolic characterization of plant protoplasts. This method lays the foundation for plant cell metabolic phenotype studies enabling comparative studies at cellular level among cultivars, species, wild-type organisms, and genome-edited plants.

Keywords Phenotype microarray, Protoplasts, Plant cells, Metabolic activity, Cellular level phenotyping

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Background

The selection of productive plants suitable for different environmental conditions is guided by genetics, epigenetics, environmental pressures and, in the case of farming, crop management. Necessarily, all these factors shape plants phenotypes. Indeed, the interaction between genotype and environment influences plant's growth and development, affecting structural traits at cellular, tissue and organ level, as well as physiological traits determining plant functioning. This, in turn, affects tissue development, organ formation, yield performance and the overall morphology of the plant [1].

Among *-omics* methods, phenomics offers practical approaches for analyzing multidimensional phenotypic information, which is essential for understanding and predicting the genetic potential of agriculturally important organisms. Plant phenomics combines several disciplines as plant biology and biotechnology, agronomy, mathematics, engineering, and data analysis, which allow to explore the most complex plant traits such as growth, development, tolerance, resistance, structure, physiology, ecology, and the basic measurements of quantitative parameters laying the basis for complex and multifactorial traits assessment [2].

Although plant phenotyping offers insights at whole organism level through the study of dynamics resulting from plant – environment interactions, nowadays the cellular-level characterization is becoming essential for identifying and dissecting the metabolic basis of different phenotypes, and the impact of genetic-related metabolic modifications. Indeed, over the past two decades, advancements in novel sensors, automation, and quantitative data analysis have significantly enhanced phenotyping, enabling high-capacity analyses across multiple scales, from entire agricultural landscapes to cellular structures. Nevertheless, there is still a lack of a fast, reproducible, and high-throughput method for comprehensive plant cellular phenotype analysis that also quantifies the phenotype as a numerical value [3, 4].

The physiological state of a cell is a valuable indicator for characterizing its behavior in a specific environment, indeed, plant cell suspension cultures are widely used so far in plant biology as an effective tool for investigating various phenomena avoiding structural complexity of whole plants [5, 6].

Unlike intact plants, which can mitigate stress effects through interactions between organs and tissues, cell cultures lack such mechanisms, allowing direct experimental access [7]: uniformly surrounded by the medium, cells experience homogeneous conditions, facilitating stress control and the analysis of their metabolic behavior [8] without morphological interference [9].

Their homogeneous in vitro cell populations, abundant material availability, rapid growth rates, and reproducible conditions make them ideal for several application, such as production of plant-specific secondary metabolites [10], molecular farming [11, 12], analyzing complex physiological and developmental processes at both cellular and molecular levels so far [13].

Transcriptomics, proteomics and metabolomics have been largely used to define cellular responses at molecular level to environmental conditions. In particular, metabolomics [14, 15], which allows the large-scale analysis of small molecules produced as intermediates or end-products of all metabolic processes, is widely used in plant science. While it provides a detailed and accurate description of cellular responses to various conditions, the procedures for sample preparation, data acquisition, and analysis are time-consuming and often labor-intensive. Moreover, despite its depth of analysis, metabolomics alone may not always capture the full dynamics of metabolic activity in real time. Likewise, transcriptomics, analyzing gene expression and providing insights into responses to different conditions, also has its limitations. While it can offer valuable information about cellular responses, it is often less effective in providing an immediate and comprehensive picture of metabolic dynamics.

Bochner et al. [16] were the first to describe cell levels phenomics with quantitative output using Phenotype Microarray (PM) technology, where the active metabolism was continuously monitored and translated into numerical values. This approach laid the groundwork for advancements in cellular analysis, as it enables the simultaneous method for assessing cellular metabolism under differing conditions, enabling real-time results and allowing for the analysis of a greater number of conditions more efficiently. Indeed, PM is a high-throughput technology developed by Biolog™ for metabolic characterization studies at the cellular level, and it is based on colorimetric reactions of tetrazolium dyes occurring during cellular respiration on different substrates. PM technology can highlight differences in growth requirements, nutrient utilization, chemical sensitivity, and genetic diversity in bacteria [17–20], fungi [21], entire microbial communities, [22], and mammalian cells [23]. The application of PM to plant cells would allow both the comparison of phenotypes of different cell lines and the elucidation of phenotypes controlled by specific genes through the comparative analysis of mutant and wild-type.

Notably, a protocol for plant cell characterization has not yet been developed due to difficulties in identifying a suitable reporter of cell activities and the presence of cell wall [9]. The latter, in fact, causes an accumulation of the dye inside the cells, implying the need to extract it with a cost- and labor-intensive procedure to verify cellular metabolic activity. In this case, the use of plant protoplasts represents a viable and effective approach for several applications [24–26]. They preserve their cellular identity [27] but with an unknown epigenetic status, since their histone modifications occurred almost immediately after stress applications [28]. Nevertheless, they retain many metabolic pathways, physiological and cellular functions of a whole plant cell [27], allowing them to be used as a valid alternative to cell suspensions. Therefore, analyzing protoplast metabolism can provide insights into essential biochemical processes for understanding plant cells activity and, by extension, predicting the overall metabolic functions of the whole plant organism [29]. In particular, the main features that make protoplasts suitable for metabolic screening are: (i) *cell simplicity,* since protoplasts provide a simplified system to study cellular processes avoiding complexities introduced by cell wall and offering the advantage to study complex growth properties [30], (ii) *direct access to cellular components,* as the lack of cell wall makes it easier to manipulate, studying dedifferentiation process [27, 31] and intracellular environment [32], making the introduction and the measurement of substances more effective, (iii) *inter-homogeneity*: protoplasts isolated from the same tissue of the same donor plant are generally highly homogeneous cell population in terms of metabolic phenotype [27], largely used for modern molecular biology and epigenetic methods [26]. This helps in reducing variability in experimental results leading to more consistent and reproducible data, (iv) *response to stimuli:* protoplasts can respond to various environmental and chemical stimuli while preserving the characteristics of cell cultures, providing insights into how whole plants might react to similar conditions; v) *genetic manipulation:* protoplasts are easier to be genetically modified compared to *in toto* plants, enabling the study of specific genes and their roles in metabolism [33], and allowing for predictions about the overall metabolic responses or adaptations [25, 34, 35], (vi) single-cell application in *-omics* science [36] and ultimately, (vii) *rapid screening* [37, 38] of metabolic responses to various treatments, making the analysis faster and more efficient than using whole plants. Nowadays, protoplasts can be genetically engineered for antibody production through plant molecular farming, offering a cost-effective and scalable alternative to mammalian systems, and also serving as a vital tool in pathology for isolating and studying viruses, representing a straightforward, adaptable, and ethical alternative to conventional model systems [39]. Furthermore, protoplasts also serve as research platform for developing biomolecular assays to investigate signaling mechanisms related to biotic and abiotic stress [40–42].

Additionally, protoplasts are largely employed to validate sgRNA activity before proceeding with for DNA-free genome editing or using the *Agrobacterium*-mediated stable transformation of callus. Developing a reproducible and efficient method to assess protoplast metabolic activity is essential for validating genetic engineering techniques, such as CRISPR/Cas9 modifications [43]. This is particularly important in the context of plant-protoplast transient transformations [44], which enable rapid molecular-level assessments with relevance to pharmaceutical and biochemical applications [41]. In this study, the development, testing, and first utilization of PM technology of Omnilog (Biolog™, US) instrument for the characterization of metabolic phenotypes in plant protoplast was reported. Indeed, the PM could be a highly valuable method also for plant phenomics research as it allows a fast and high-throughput analysis of metabolic responses under various conditions. It could help to identify metabolic biomarkers linked to specific metabolic traits, provide insights into plant stress responses, adaptation, and resilience, that could be crucial for improving crop sustainability and tolerance to environmental stress. Additionally, PM can be integrated with other *-omics* technologies, offering a comprehensive view of plant physiology.

Here, PM was used in combination with the Alamar blue (AB) dye [45]. AB dye is a widely used colorimetric and fluorometric method to assess real-time cell viability, proliferation, and cytotoxicity in various cell types [46], primarily mammalian cells and microorganisms, but also including plant cells. Its principle of action is based on the ability of the active component resazurin, a blue, non-fluorescent redox dye, to act as an oxidation–reduction indicator. Metabolically active plant cells reduce resazurin to resorufin, a pink, highly fluorescent compound, through enzymatic activity. This color and fluorescence change indicates cell viability and can be measured spectrophotometrically or fluorometrically. Vice versa, dead or inactive cells do not reduce resazurin, resulting in minimal color change.

After identifying the most suitable redox marker as an indicator of cell activity and optimizing the conditions for PM testing on protoplasts isolated from *Solanum tuberosum L.* leaves, we extended the metabolic analyses to *Solanum lycopersicum L.* protoplasts, evaluating the genus response to different NaCl concentrations, which

serves as a common environmental stressor [47], and to specific toxic compounds. Our findings demonstrate that the standardized high-throughput method developed is effective for the metabolic characterization of plant protoplasts, establishing a foundation for plant cell phenotyping studies.

Methods

Chemicals and equipment

All chemicals were purchased from Sigma-Aldrich, Duchefa Biochemie and Merck (Additional file 1). Equipment used in this study are listed in Additional file 2.

Plant material and in vitro growth conditions

Solanum tuberosum (cv. Morella) plants were propagated from existing in vitro cultures maintained in the germplasm collection laboratory of Department of Agricultural, Environmental, Food and Forestry Science and Technology (University of Florence, Italy) [48]. Portions of stem, containing at least one internode, under laminar flow hood and sterility conditions were cut. The explants were then transferred into Magenta™ vessels containing Murashige and Skoog (MS) Basal Medium [49], Sigma-Aldrich, composed by 2.0 mM KNO_3, 1.6 mM NH_4NO_3, 0.2 mM $CaCl_2{\cdot}2H_2O$, 1.0 mM $MgSO_4{\cdot}7H_2O$, 0.1 mM KH_2PO_4, 0.1 mM $FeSO_4{\cdot}7H_2O$, 0.1 mM $Na_2EDTA{\cdot}2H_2O$, 5.0 µM H_3BO_3, 0.5 µM $MnSO_4{\cdot}H_2O$, 0.1 µM $ZnSO_4{\cdot}7H_2O$, 0.1 µM $CuSO_4{\cdot}5H_2O$, 0.1 µM $NaMoO_4{\cdot}2H_2O$, 0.1 µM $CoCl_2{\cdot}6H_2O$, 0.5 mg/L Thiamine-HCl, 0.5 mg/L Pyridoxine–HCl, and 0.1 mg/L Nicotinic acid) supplemented with 25 g/L sucrose and 2 g/L phytagel, adjusted to pH 5.7, for growth and root induction [50]. The vessels were incubated in a climate chamber at 22 °C with 70% humidity and a 16-h light/8-h dark photoperiod, with a light intensity of 150 µmol m^{-2} s^{-1}. *Solanum lycopersicu*m (var. *cerasiforme*) seeds were sterilized with 70% ethanol for 30 s, followed by 20% hypochlorite for 10 min. They were then washed five times with sterile distilled water and placed in sterile vessels containing MS medium for growth. In this preparation, no phytohormones were used. The seeds were kept in the dark in the same climate chamber for four days until germination.

Protoplasts culture medium

MS medium, free of NH_4NO_3, was prepared containing vitamins (glycine-free base, 2.0 mg/L; myo-inositol, 100.0 mg/L; nicotinic acid-free acid, 0.5 mg/L; pyridoxine·HCl, 0.5 mg/L; thiamine·HCl, 0.1 mg/L), along with 50 g/L glucose and 25 g/L mannitol [51, 52]). The medium had an osmolarity of 0.600 osmol/kg, and the pH was adjusted to 5.6. It was then sterilized by autoclaving at 121 °C for 21 min. In this preparation, no phytohormones were used [53].

Protoplasts isolation

20-days-old leaves of in vitro raised *S. tuberosum* (cv. Morella) and *S. lycopersicum* (var. *cerasiforme*) were cut into fine strips (1–2 mm) using the sharp blade, soaked in 20 mL of the Basic Solution (composed by 10 mM $CaCl_2$, 0.2 mM KH_2PO_4, 1 mM KNO_3, 1 mM $MgSO_4$, 5 mM 2-(N-morpholino) ethane sulfonic acid [MES], 1% (w/v) of polyvinyl pyrrolidone 10; pH 5.6) and incubated at 22 °C in the darkness for 20 min in a rotary shaker at 100 rpm, as described in [52]. The Basic Solution was then replaced with 20 mL of the Enzyme Solution, which was composed by cellulase 1% [w/v] and macerozyme 0.24% [w/v] dissolved in the Basic Solution and set to 0.600 osmol/kg osmolarity with mannitol. Leaf strips were incubated overnight [35, 52] in the darkness at 22 °C with gentle shaking (50 rpm) for 16 h. Undigested tissues were removed by filtering the sample through 100 µm vacuum cell strainers and washing it with 10 mL of the Washing Solution W5 (composed by 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, 155 mM NaCl; pH 5.6). Protoplasts were collected by centrifugation at 70 ×g for 10 min at room temperature. The resulting pellet was resuspended in 8 mL of Sucrose Solution (15% [w/v] of sucrose dissolved in the Basic Solution) and centrifuged again under the same conditions. A distinct protoplast layer and was carefully transferred to a new tube. The protoplasts were then washed twice with Washing Solution W5, each time centrifuging at 70 ×g for 10 min at room temperature. After resuspension in 10 ml of Washing Solution W5, the protoplasts were counted under an optical light microscope (Polyvar Reichert-Jung) with a Bürker chamber [54]. To discriminate viable cells, protoplasts were stained with trypan blue (0.04% [w/v]), which selectively stains dead cells blue by binding to intracellular proteins in those with compromised membranes. This criterion for distinguishing live cells, works by identifying the blue-stained dead cells, thereby excluding them from the analysis.

The final protoplasts density was resuspended in protoplasts culture medium and adjusted to 10^6 and/or 10^7 protoplasts/mL, depending on the utilization.

Phenotype microarray

PM technology by Biolog, Inc.™ uses redox potential indicators as reporters of cellular metabolic activity, and 96-well precasted microplate (PM1-20) in which different compounds are placed on the bottom of

different wells to test different phenotypes (utilization of different nutrients, sensitivity to pH, to osmotic stressors and toxic compounds). Redox potential indicators are compounds that change color depending on their oxidation or reduction state, reflecting the oxidation state in cells. Redox potential indicators in PM react to cellular respiration, resulting in a color change that serves as a proxy for cellular metabolic activity. Biolog™ has developed different redox potential indicators (Dye Mixes) suitable for different types of microorganisms and mammalian cells characterized by being uncolored or slightly colored in the oxidized form and dark colored (violet) in the reduced form. Metabolically active cells reduce the oxidized form of the dye causing the culture medium to change from uncolored to colored (violet). The Omnilog instrument is equipped with a CCD camera that records the image of the PM microplates during time. The Biolog™ software converts the color detected in each well of the PM microplates to grayscale (ranging from dark gray, indicating high consumption, to light gray, indicating low consumption) and expresses the color intensity as a numeric value, the Arbitrary Omnilog Unit (AOU). Normally, Biolog™ dyes turn from uncolored (or lightly colored) to dark colored by metabolic active cells, and kinetic curves that increase with time are generated. The innovative dye used in this work, turns from dark purple to uncolored, generating kinetic curves that decrease with time.

In this study, nine redox potential indicators commercialized by Biolog™ and the innovative dye AB [45] were tested on *S. tuberosum* and *S. lycopersicum* protoplasts. Biolog™ has patented dyes suitable for different cellular type, as described in PM Biolog™ protocols. According to these, Biolog™ dyes can be used for bacteria (Dye A for gram negative bacteria [55], Dye F and G for fast and slow growing gram positive bacteria, respectively [56], Dye H for both gram positive and negative bacteria, Dye E and D for fungi [57], Dye MA, MC and MB for mammalian cells [58]. Although Biolog™ dyes are indicated for specific uses, they lack high specificity, as dyes recommended for particular cell types can also be effectively used for others [59–61]. Therefore, their effectiveness as reporters of protoplast metabolic activity warrants further investigation. In light of this, we tested the following Biolog Dyes on protoplasts: Biolog Redox-Dye Mix A (100x), Biolog Redox-Dye Mix D (100x), Biolog Redox-Dye Mix E (100x), Biolog Redox-Mix F (100x), Biolog Redox-Dye Mix G (100x), Biolog Redox-Dye Mix H (100x), Biolog Redox-Dye Mix MB (6x), Biolog Redox-Dye Mix MC (6x). Biolog dye Mix A, D, E, F, G, H and MB and MC were diluted 100-fold in protoplast suspensions, as suggested by manufacturing company.

The innovative dye AB was tested at different concentrations to find the dose that maximizes the height of the metabolic activity curves. Indeed, after the acquisition of the PM plate images, the color of the wells was transformed to a grey scale quantified as AOU. The starting blue of oxidized AB was detected as dark grey (high AOU values), while the final pink of reduce AB was detected as light gray (low AOU value). To identify the AB dose that maximize the metabolic activity in protoplast suspension, different concentrations were tested. AB dye was prepared as a 0.2% (w/v) stock solution, sterilized by filtration and tested at the following concentration (w/v): 0.0005%; 0.001%; 0.004%; 0.008%; 0.012%. All dye

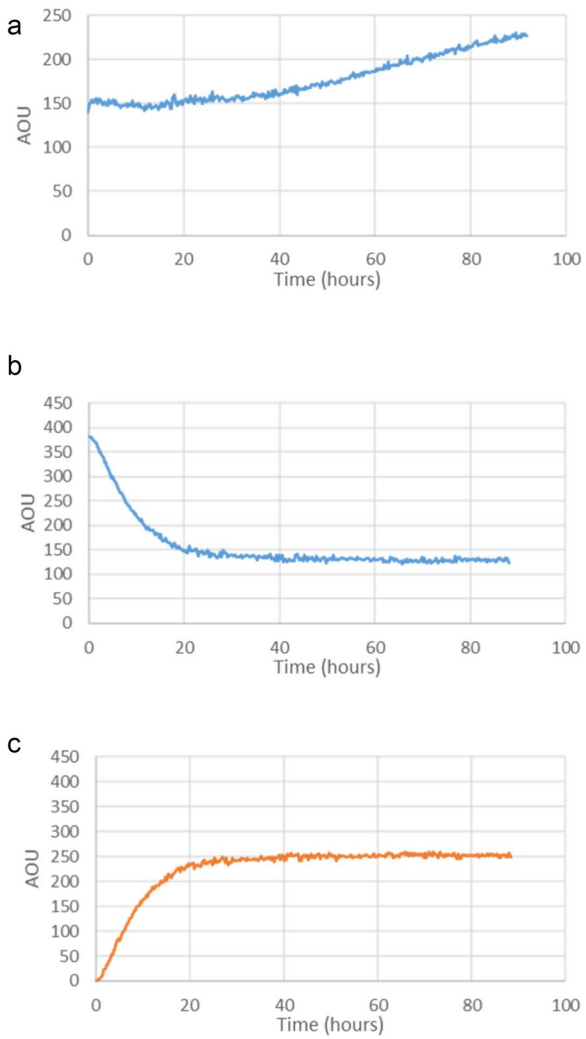

Fig. 1 Kinetic curves of color variation produced by *Solanum tuberosum* in presence of Dye Mix MC (Biolog™) and AB. Kinetic curves obtained in presence of Dye Mix MC (**a**) and 0.004% (w/v) AB (**b**) as recorded by the OmniLog software. The kinetic curve based on AB as a metabolic reporter (**b**) has been transformed to an increasing curve (**c**), as described in the text

suspensions were added in triplicate in the wells of a 96 well microplate (250 µL per well).

Since the kinetic curves obtained with Biolog™ dyes and AB dye respectively increase and decrease with time (Fig. 1, a and b, respectively), to have comparable data, the kinetic curves produced by AB were transformed in increasing curves by subtracting the AOU value measured by Omnilog instrument and detected at each time, to the AOU value recorded at the beginning of the incubation (t = 0) (Fig. 1, c). The value obtained from this subtraction was renamed Metabolic Activity Index (MAI), which in this case was understood as a quantitative measure used to evaluate the physiological state of protoplasts over time. In this study, the MAI reflects the persistence of protoplasts in different conditions, providing an indicator of their metabolic resilience.

First, to evaluate the ability of each dye to measure the metabolic activity of the protoplasts, the percentage variation (%V) among initial and final value was calculated as:

$$(\%V) = (H_{92} - H_0 / H_0) * 100$$

where H_{92} is the height of the curve at 92 h of incubation and H_0 is the height of the curve at the beginning of the incubation period (t = 0). Remarkably, after 92 h of incubation, the protoplasts remain in their wall-free state without regenerating the cell wall, especially when present in high concentrations [53].

For the evaluation of *Solanum* genus plants response to NaCl, the following concentrations were tested: 10 mM, 25 mM, 50 mM, 75 mM, 100 mM, 125 mM, 150 mM, 200 mM.

The kinetic curves determined by AOU for Biolog™ dyes and calculated for AB dye, allowed the calculation of the Areas Under the Curve (AUC) for each NaCl concentration, enabling to condense their kinetics into a computable number providing a straightforward way to quantify changes over time. AUC was estimated via the trapezoidal rule, a numerical method that approximates the integral of a function. This method is particularly useful for estimating the area under a curve when the function is not easily integrable analytically. First, the AUC is divided into a series of trapezoids which are formed between two adjacent time points on the curve.

For each pair of adjacent points, the area of the trapezoid was calculated. Then, the total AUC was obtained by summation the areas of all individual trapezoids [62].

Every AUC value was then utilized for the calculation of the Activity Delta, which expressed the ratio among the AUC traced by the metabolic activity at a determined NaCl concentration and the AUC traced by the metabolic activity in control condition (0 mM NaCl).

Sensitivity of protoplast to different toxic compounds was tested by a subset of Biolog™ PM chemical sensitivity panels (PM11-20). Indeed Biolog™ produces 96-well plates for chemical sensitivity analysis of microorganisms containing a wide range of toxic compounds (https://www.biolog.com/products/metabolic-characterization-microplates/microbial-phenotype). Each plate contains 24 different toxic compounds at 4 different concentrations, distributed over four adjacent wells, from the lowest to the highest. Although originally designed for the microorganisms, their use for the study of protoplasts must be assessed, as they contain some compounds that can also give toxic effects in plant cells (toxic anions and cations, antifungals, antimetabolites, etc.). As a first attempt to apply preconfigured Biolog™ 96-well plates and considering the potential interest in measuring plant protoplast metabolic activity, a subset of PM microplates for chemical sensitivity analysis, including those with documented activity on plant cells and others without, were selected. In particular, PM11C, PM15B, PM18C, and PM19. Chemical sensitivity panels contained 240 toxic compounds (toxic cation and anion, anbibiotic, fungicide, disinfectants, antimetabolits, cheletros, etc.…). The compounds assayed with their related mode of action and doses, were listed in bold in Supplementary File 1.

Statistical analysis

All the statistical analyses were carried out in the RStudio environment (v 4.4.1) (R Core Team, 2022). Prior to each analysis, the Shapiro–Wilks test was applied to evaluate data distribution.

Then, statistical differences were assessed through two-way ANOVA or Kruskal–Wallis tests using *stats* (v 4.1.2) and *ggpubr* (v 0.6.0) packages, respectively for normal or not-normal distributed data. For multiple comparisons among redox marker dyes, the post hoc Dunn test, with the Benjamini–Hochberg correction, were performed. For each protoplast's concentrations used, differences in the summary value of Metabolic Activity Index (MAI) were fulfilled among *S. lycopersicum* and *S. tuberosum* protoplasts on different concentrations of NaCl at the incubation time of 25, 50,75 and 92 h with post hoc Dunn Test or Tuckey test. Differences in Activity Delta of *S. tuberosum* and *S. lycopersicum* protoplasts on different NaCl concentrations were calculated with ANOVA followed by a Tukey post-hoc test.

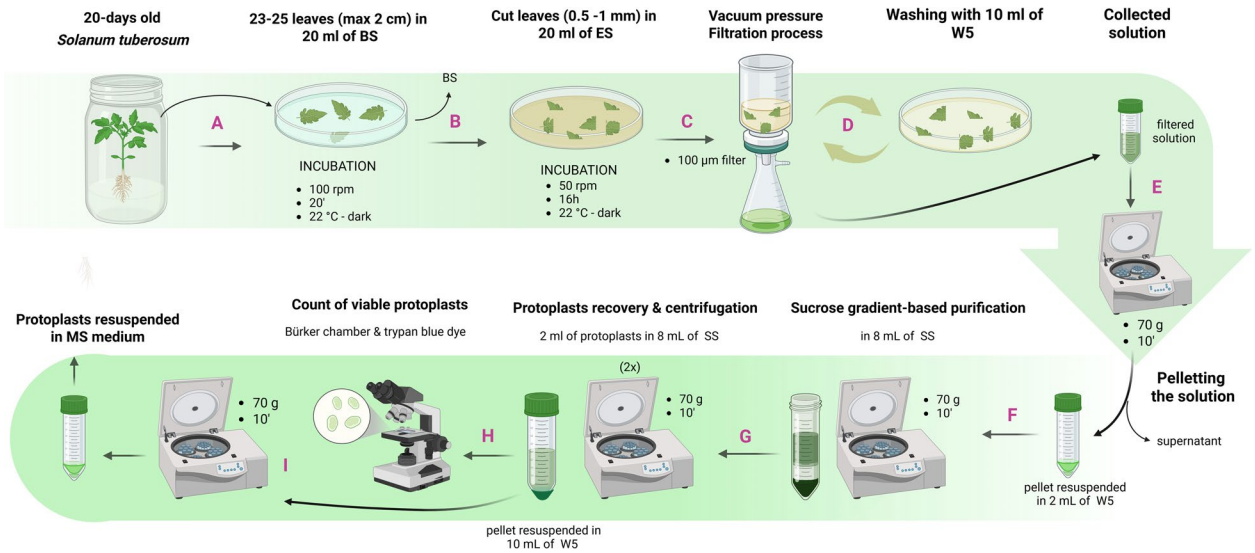

Fig. 2 Components and passages of the research protocol set-up and used in this work

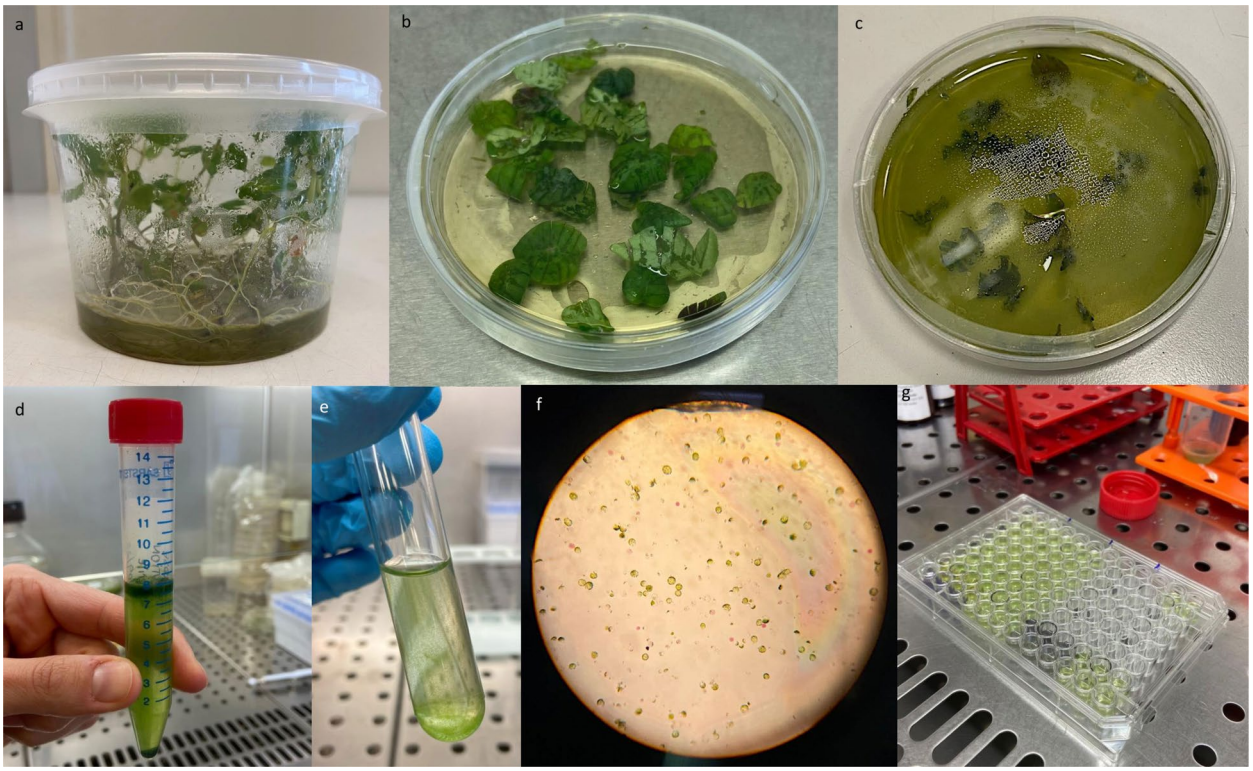

Fig. 3 Protoplasts isolation from *Solanum tuberosum* leaves. **a** Two weeks old plant of *S. tuberosum* cultivated *in-vitro,* grown at 23 °C under long day (16 h light/8 h dark) conditions; **b** *S. tuberosum* leaves cut with horizontal cut in basic solution; **c** Protoplasts isolation in enzyme solution after 16 h of incubation; **d** Overlaying protoplast solution during sucrose density gradient-based protoplast purification; **e** Protoplasts recovery and treated with washing solution during centrifugation steps; **f** Light microscope image observed at 40X magnification of isolated protoplasts; **g** microplate inoculated with protoplasts suspension and markers of redox potential

Results

Protoplast isolation and cell wall digestion protocol optimization

The complete protocol involves a meticulous two-day process, illustrated in Figs. 2 and 3) On the first day, at least 16 h before the start of the second day protocol, 20 mL of the Basic Solution was placed into a glass Petri dish. Approximately, 25 mature leaves from twenty days old micro propagated *Solanum* plants (Figs. 2 and 3a, S. *tuberosum* leaves) without the leaf stalk, and reaching 0.5 g, were submerged in the solution, ensuring that all brown parts were removed (Fig. 2, step A). The glass Petri dish was then incubated in darkness at 100 rpm for 20 min at 22 °C, under the chamber conditions described in the Materials and Methods section. After incubation, 15 mL of the Basic Solution was removed using a pipette, and the leaves were then sectioned into smaller pieces (1–2 mm in size) (Fig. 3b, Fig. 2 step B). The remaining solution was completely removed, and 20 mL of the Enzyme Solution, previously stored at −20 °C, was added. The same enzyme mixture was used for both plant species after confirming proper cell wall degradation, which was assessed by visually evaluating the roundness of the protoplasts. Furthermore, this enzymatic mixture enabled optimal recovery and high-yield isolation of protoplasts in both tested species. The mixture was then incubated overnight with agitation at 50 rpm, in the dark, at T 22 °C (Fig. 2, step B).

Optimal incubation time in the Enzyme Solution was crucial for efficient protoplast isolation [63]. According to our protocol, the best material consisted of 23–25 fresh leaves from 20-day-old in vitro-grown plants, collected from the upper part of the plant and not exceeding 2 cm in size (Fig. 2, step A). The leaves were cut into 0.5–1 mm sections perpendicular to the central vein and incubated in 20 mL of Enzyme Solution for 16 h (Fig. 2, step B), ensuring a yield of at least 10^6 protoplasts/mL. Shorter incubation times (< 14 h) resulted in incomplete cell wall degradation.

On the second day (Fig. 2, step C), a 100 µm filter was placed into the lid of a magenta vessel. Small leaf pieces were added to the filter, and vacuum pressure was applied while swinging the lid for one minute to facilitate filtration. The filtered solution was then collected into a tube. The leaves were thoroughly washed in 10 mL of Washing Solution W5 in a sterile Petri dish, and the filtration process was repeated until the flow-through appeared pale green (Fig. 2, step D). All filtered solutions were pooled together and centrifuged at 70 ×*g* for 10 min (Fig. 2, step E). The supernatant was quickly discarded, and the pellet was resuspended in 2 mL of Washing Solution W5 by gently inverting the tube.

The filtered solution containing protoplasts was then layered into 8 mL of the Sucrose Solution and centrifuged for 10 min at 70 ×*g* (Fig. 2, step F). Following centrifugation, the formation of a dark green phase containing protoplasts was expected. The height of this phase varied but was always positioned in the middle of the Sucrose Solution, making it easily identifiable (Fig. 3d).

Two milliliters of the protoplast phase were added to 8 mL of Washing Solution W5 in a new tube and gently inverted to mix. The mixture was then centrifuged for 10 min at 70 ×*g* (Fig. 2, step G). Afterward, 8 mL of the supernatant was replaced with 8 mL of fresh Washing Solution W5, and the tube was gently inverted again. The centrifugation step was repeated once. The supernatant was discarded, and the pellet was resuspended in 10 mL of Washing Solution W5 (Fig. 3e and Fig. 2, step G). Total and viable protoplasts (calculated by exclusion) were counted under a light microscope (Fig. 3f) using a Bürker chamber and trypan blue dye, and the number of cells was determined (Fig. 2, step H). Finally, the solution was centrifuged again for 10 min at 70 ×*g*. The supernatant was removed, and the pellet was resuspended in the amount of MS medium calculated to obtain concentrations of 10^6 and 10^7 protoplasts/ml, respectively (Fig. 2, step I). Then, 100 µl of each suspension was plated on Luria–Bertani medium to check for possible microbial contamination. Despite the critical steps involved in protoplast isolation, such as the age and conservation of donor plant material, the conditions of enzymatic incubation, the composition of the solutions, and the frequent physical damage that protoplasts can suffer (for example, during centrifugation), careful attention to details in these process allows for a high level of reproducibility in both the yield and viability of protoplast isolation, as well as in the subsequent metabolic characterization, which is the focus of the study.

Identification of optimal redox marker dye for Phenotype Microarray analysis of *Solanum tuberosum* protoplasts

In this study, Biolog™ dyes were evaluated as redox potential indicators for protoplasts. Additionally, AB, a compound traditionally used to assess protoplast metabolic activity, was also examined. Differently from Biolog™ dyes, AB is dark colored in oxidized form (blue) and light pink in the reduced form, thus an inverse kinetic curve (decreasing with time) is expected for active cells in the PM system.

To test redox potential indicators as reporters of protoplast metabolic activity, protoplast isolated from leaves of model plant *S. tuberosum* were resuspended in MS (10^6 cells/ml and 10^7 cells/ml) and stored overnight

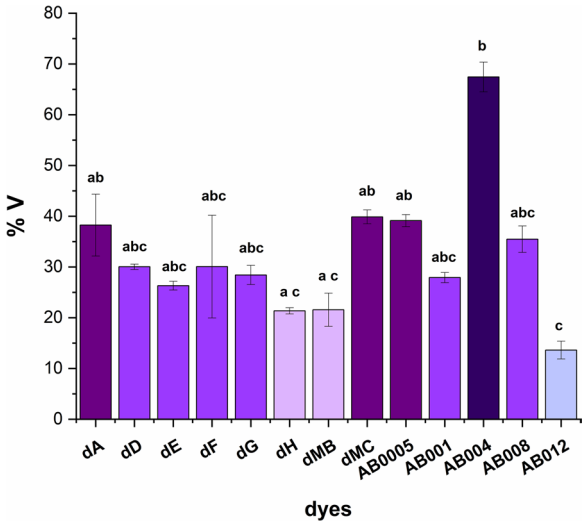

Fig. 4 Selection of the most efficient redox marker dye comparing Biolog™ validated dyes and the AB dye at different concentrations. Percentage Variation (% V) of redox marker dyes consumption. % V was calculated among initial and final (at 92 h) values of each dye consumption. Different superscript letters indicate results of Dunn test's post-hoc among different dyes following the Kruskal–Wallis test (p = 0.00088). Legend: *dA* = Biolog™, *dyeA*; dD = Biolog™ *dyeD*; dE = Biolog™ *dyeE*; *dF* = Biolog™ *dyeF*; dG = Biolog™ *dyeG*; dH = Biolog™ *dyeH*; dMC = Biolog™ *dyeMC*; *dMB* = Biolog™ *dye*; MB; AB0005 = [0.0005%]; *AB001* = AB[0.001%]; *AB004* = AB[0.004%]; *AB008* = AB[0.008%]; *AB012* = AB[0.012%].

in the dark. They were then combined with various dyes and distributed in triplicate into the wells of a 96-well microplate (250 µL per well). AB stock solution (0.2% [w/v]) was added to protoplast suspensions to achieve final AB concentrations of 0.0005%, 0.001%, 0.004%, 0.008%, and 0.012% (w/v). The protoplasts were then distributed in triplicate into the wells of a 96-well microplate (250 µL per well). The microplate was incubated in the Omnilog at 25 °C for 92 h., and kinetic curves of color change were recorded for each well.

Statistical differences among different Biolog™ dyes used were observed (p = 0.00088). Biolog™ dyes (dA, dD, dE, dF, dG, dH, dMC, dMB) achieved an average maximum value of 35% V, with dMC reaching the highest value at 40% V. In contrast, AB, tested at different concentrations, exhibited a higher average %V, with the peak value observed at a concentration of 0.004% (w/v) (Fig. 4), where it reached 67% V. Overall, these findings suggested that Biolog™ dyes used according to the manufacturer's guidelines, are not fully suitable for PM analysis of *S. tuberosum* protoplasts, whereas AB at 0.004% (w/v) proves to be an effective alternative.

Protoplast concentration for assessing metabolic activity

A crucial point in PM experiment is the standardization of the inoculum. To guarantee data reproducibility and ensure comparability across different experiments, the initial cell density in the PM plates must be standardized to a specific value. The concentration should be sufficient to obtain a high kinetic curve in the non-stressed state (positive control), and to detect a reduction of the curve under stress conditions (i.e. in the presence of an osmotic stressor or other toxic compounds). Therefore, the activity of different concentrations of *S. tuberosum* protoplasts (10^6, 10^5, 10^4, 10^3, 10^2 cell/mL) was evaluated in MS with AB 0.004% (w/v) (Fig. S1). The percentage variation (%V) was calculated among initial and final (at 92 h) values of the kinetic curves detected by the OmniLog software. The highest %V recorded was obtained for protoplasts at 10^6 cells/ml, reaching 178.319. Contrarily, a huge reduction was observed when protoplasts were reduced to 10^5 cells/ml, reaching a value of 2.45% V. The %V recorded continued to decrease as the protoplast concentration decreased, resulting in values of 5.7, 3.19, 17.90 for 10^4 cells/ml, 10^3 cells/ml and 10^2 cells/ml, respectively. These results indicate that a starting protoplast density of 10^6 cells/mL is optimal for PM assays. Higher concentrations would require processing a significantly larger number of leaves, yet successful isolation remains uncertain due to the inherent challenges of protoplast handling. Whenever feasible, the authors conducted metabolic tests at higher protoplast concentrations.

PM analysis of NaCl-induced stress in protoplasts of *S. tuberosum* and *S. lycopersicum*

After determining the optimal protoplast concentrations and the most effective redox potential marker, metabolic activity assessments in the presence of different concentrations of NaCl were also conducted on protoplasts isolated from another crop species within the same genus, *S. lycopersicum*. NaCl normally serves as a common environmental stressor that significantly impacts the growth and metabolic activity of plants, particularly in agricultural settings where salinity stress is prevalent.

The activity of *S. tuberosum* and *S. lycopersicum* protoplasts at the concentrations 10^6 and 10^7 cells/mL was evaluated in MS medium with 0.004% (w/v) AB at increasing concentrations of NaCl (Fig. 5, Table S1). In particular, the protoplasts were tested in the absence of NaCl (which stands for the Control), and in the presence of 10, 25, 50, 75, 100, 125, 150 and 200 mM NaCl. In this case, the metabolic activity was assessed based on protoplasts' persistence under increasing NaCl concentrations. It was expressed as Metabolic Activity Index (MAI) over

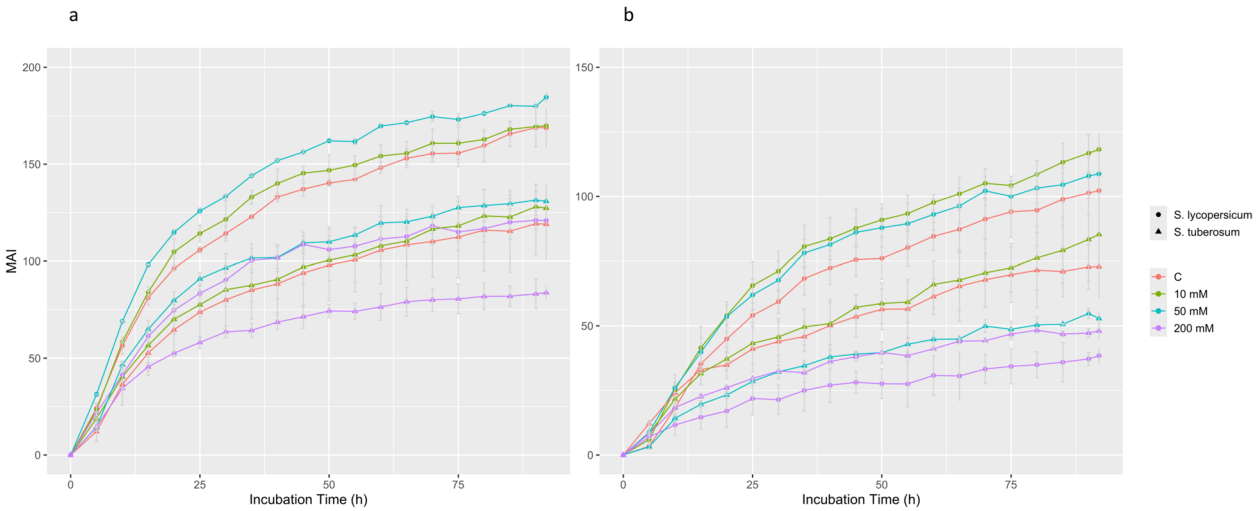

Fig. 5 Persistence Kinetic of 10^6 (**a**) and 10^7 (**b**) protoplasts/mL of *S. lycopersicum and S. tuberosum* on different concentration of NaCl. Persistence is expressed in Metabolic Activity Index (MAI) over time (from T0 to T92 h) and was determined by AB004 consumption

time (from T_0 to $T_{92\ h}$), which evaluate the physiological state of the cells, and particularly their ability to persist and remain under specific conditions.

The persistence kinetics observed at the two tested protoplast concentrations were species-specific, with consistently and significantly higher MAI values recorded at a concentration of 10^6 protoplasts/mL across all tested NaCl concentrations (Fig. 5a). Nevertheless, the same trend of persistence in NaCl, seems to be maintained in both the protoplasts concentrations tested (Fig. 5 a and b), highlighting a clear decrease of MAIs in 200 mM NaCl. Interestingly, protoplasts isolated from the two different species, while maintaining a shared growing trend over time, showed a different reaction in presence of 10 mM and 50 mM NaCl: the trend observed in *S. lycopersicum* reached significantly higher values of MAI (160–180 MAI at 10^6 cells/mL, and 100–120 MAI at 10^7 cells/mL), in both protoplasts concentrations, and with 0, 10 and 50 mM NaCl. Contrarily, *S. tuberosum* seemed to be less able to survive in the presence of NaCl, showing kinetics that remained significantly lower than those of *S. lycopersicum* under all conditions. These results align with existing literature, which reports a greater salt tolerance in tomato plant [64]. Therefore, the system

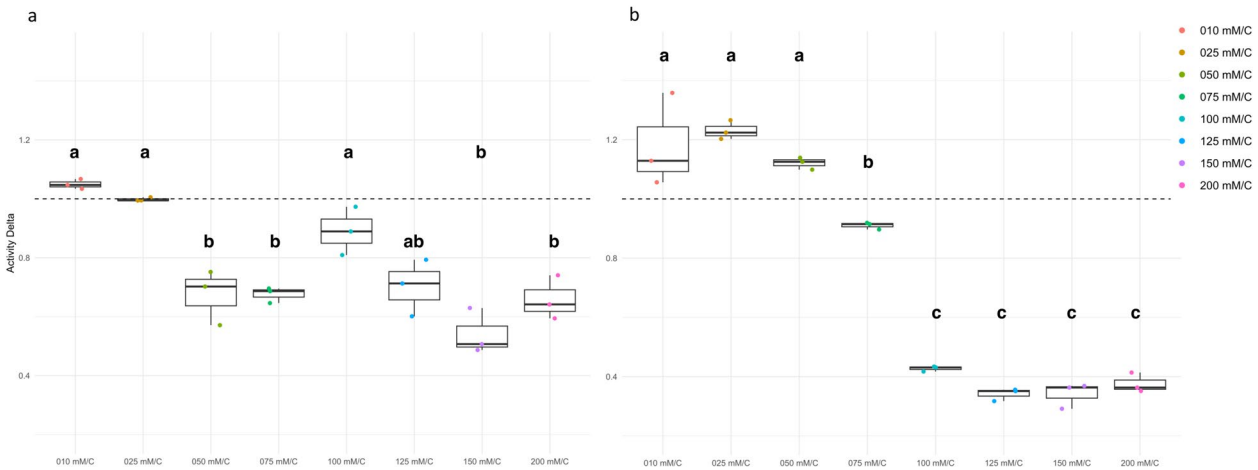

Fig. 6 Activity delta of *S. tuberosum* (**a**) and *S. lycopersicum* (**b**) protoplasts (10^7 cells/mL) on different NaCl concentrations, compared to the control (C = 0 mM NaCl). The letters above the bars indicate statistically significant differences between groups, calculated using ANOVA followed by a Tukey post-hoc test ($p < 0.005$). Activity Delta was calculated as the ratio between the AUC (Area Under Curve) at a specific NaCl concentration and the AUC in the absence of NaCl determined by AB004 consumption.

tested on the Biolog™ technology, and the developed protoplasts metabolism analysis set up in this study, showed a good functioning on both plant species tested, demonstrating a good ability to highlight differences in the metabolic performance.

To simplify the assessment of persistence and metabolic activity in different NaCl concentration previously reported, time-course measurements were consolidated into a single summary value. Indeed, the Activity Delta (AD), which is calculated as the ratio among the AUC traced by the metabolic activity at a determined NaCl concentration and the AUC traced in control condition, was calculated for *S. tuberosum* and *S. lycopersicum* protoplasts, for both 10^6 protoplasts/mL and 10^7 protoplasts/mL concentrations (Figs S2 and 6, respectively). Here, when the AD is greater than 1, the AUC calculated at a defined concentration of NaCl is greater than the AUC calculated for the kinetic curve at 0 mM NaCl, indicating a positive role of the tested NaCl concentration on protoplast metabolism. Conversely, when the AD is less than 1, the AUC calculated at a defined concentration of NaCl is less than the AUC calculated for the kinetic curve under control conditions, indicating an inhibitory effect of NaCl on protoplast metabolism.

Testing with Biolog™ PM microplates for sensitivity analysis

During preliminary control tests (data not shown), certain compounds reacted with the dye used in PM analysis, resulting in false positives. To identify and exclude false negatives, plates were inoculated with AB-containing, protoplast-free MS medium. To further validate the system, protoplasts were introduced into the four selected plates (PM11C, 15B, 18C, and 19), avoiding wells that had produced false positives. Furthermore, these microplates contained a sufficient number of toxic compounds that did not interfere with the AB dye, allowing sensitivity determination.

In 11C plate the compounds tested (in bold in Supplementary File 1) can have a proven effect on plant cells (if indicated) or not. In particular, among these, Bleomycin, is an inducer of double-strand breaks (DSBs) that cause a non apoptopic programmed cell death in plant cells [65], the Nalidixic acid, described as an inhibitor of chloroplast replication [66], Chloramphenicol, reported to have an effect on growth and membrane structure [67, 68] and Kanamycin, frequently used for transformant plant selection [69].

In 15B plate, the compounds tested belong to different classes with a different mode of actions (in bold in Supplementary File 1). For example, the antibiotics Phleomycin and Puromycin, used as selectable marker for transformant plant cells [70] and as inhibitor of protein synthesis [71] respectively, were tested. Also, the electron transfer agent Methyl-viologen, a photosensitizing agent that can generate reactive oxygen species (ROS) causing cellular damage and oxidative stress to plant cells [72], and CCCP, an inhibitor of mitochondrial electron transport chain, that be used to induce cellular stress and to evaluate the response to mitochondrial damage [73] were tested, as well as the chemical mutagen Sodium azide [74] and the inhibitor of ribonucleotide reductase hydroxyurea, which generate DNA damage in plant cells [75].

In 18C plate, among the compounds selected (in bold in Supplementary File 1), there were the Sodium meta-arsenite, previously used to study the detoxification in plant [76], and the Sodium metasilicate, reported to enhance stress tolerance in plants by reinforcing cell walls and promoting phenolic compounds synthesis [77].

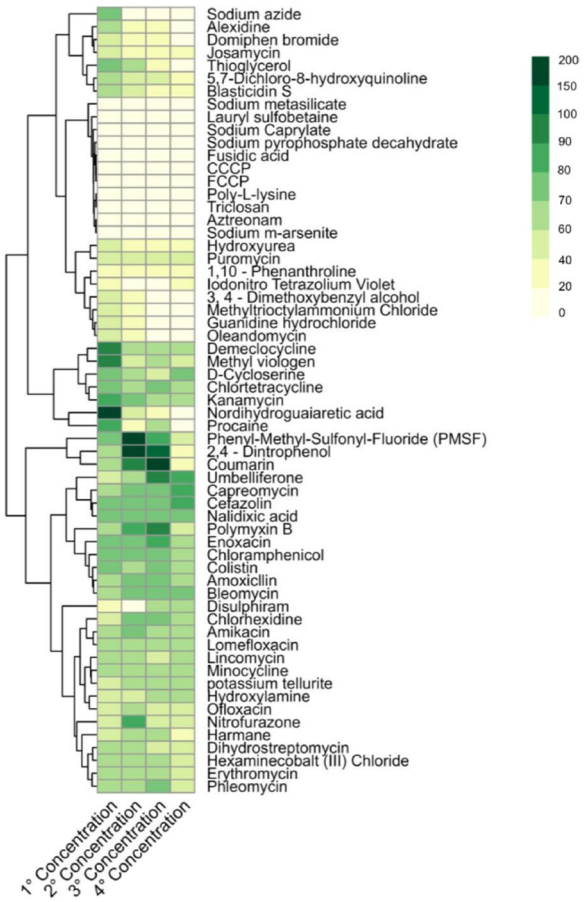

Fig. 7 Percentage variation (% V) of metabolic activity in the presence of toxic compounds. Heatmap of protoplast percentage of variation of metabolic activities on four increasing Concentration for each toxic compounds selected in PM 11C, 15B, 18C and 19. Hierarchical clustering with complete linkage of % V of different compounds was reported

In 19 plate, changes in metabolic activities of protoplasts were evaluated among all, for Coumarin, a class of organic compounds found in plants and known for their roles in plant defense, iron uptake, and interactions with microorganisms [78]. Other chemicals employed were the 2, 4- Dinitrophenol, a pollutant often used to evaluate the phytoremediation capabilities of plants [79], the Umbelliferone. a well described xenobiotic compound potentially taken up by plants from the environment [80], and, lastly, the inhibitor of the electron transport chain FCCP, that can induce cellular stress causing mitochondrial damage also in plant cells [81].

Although the %V between initial and final value (at 92 h) was minimal, a difference in metabolic response could still be observed across the different concentrations of the toxic compound (from 1 to 4, second column of Table S2): as its concentration increased, the %V decreased, indicating a less intense metabolic activity of protoplasts in the more concentrated compound (Table S2). It should be noted that Biolog™ did not disclose the exact concentrations of compounds in PM microplates.

As shown in Fig. 7, some chemical compounds resulted useful for metabolic screening of protoplasts variability (Polymyxin B, Coumarin and Umbelliferone Bleomycin, Kanamycin, Methyl viologen and 2,4- Dinitrophenol) among concentrations, suggesting that the applicated method is able discern little variation in metabolic activity of protoplasts among different substrate concentrations. Conversely, for other chemicals listed above (CCCP, Sodium azide, Hydroxyurea, Sodium meta-arsenite, Sodium metasilicate, Nalidixic acid, Chloramphenicol, FCCP, Phleomycin and Puromycin) differences among concentrations were not observed.

Discussion

The interaction between genetic inheritance and environmental conditions significantly affects the productivity and adaptability of cultivated plants, leading to the development of unique phenotypes. Within this framework, phenomics has emerged as an essential discipline, providing robust methodologies for the analysis of complex phenotypic data, which is critical for uncovering the genetic potential of important agricultural species. While traditional plant phenotyping focuses on whole-organism assessments, cellular-level phenotyping plays an essential role in dissecting the metabolic underpinnings of various phenotypes and assessing the implications of metabolic-related genetic modifications.

In this study, we applied Phenotype Microarray technology, initially developed for microorganisms and mammalian cells [16], to plant cells and in particular, to protoplasts. This high-throughput method allows metabolic characterization at the cellular level, addressing a gap in plant phenotyping protocols. The key advantage of applying PM technology to protoplast analysis is its capability to simultaneously assess hundreds of cell metabolic phenotypes within a short time, an achievement that, to our knowledge, is not feasible with existing plant cell phenotypic analysis methods. Protoplasts were largely demonstrated to be useful for advanced cellular studies providing a simple, controllable system to investigate various cellular and metabolic processes, facilitating genetic engineering procedures and enabling studies of cellular physiology [29].

In this work, the advantages of using protoplasts, such as their simplified nature, enhance the reliability of the results. Several methods and protocols for protoplast isolation have been extensively reported and refined over time [35, 82]. However, these protocols are not uniform, as they must account for various factors such as plant species, cell types, enzyme selection, physical conditions, the intended use of the protoplasts, and the developmental stage of the plant tissue. In this study, we refined a protoplast extraction protocol specifically for two relevant species, potato (*S. tuberosum*) and tomato (*S. lycopersicum*). The optimized protocol yielded the best results at concentrations between 10^6 cells/ml and 10^7 cells/ml. The feasibility of PM technology was enhanced through the use of a completely innovative dye, which is currently widely employed for the metabolic analysis of plant cells but has never before been used to track changes in their metabolism over time. Indeed, Alamar blue, at a concentration of 0.004%, can serve as an alternative to the traditional Biolog™ dyes for assessing the reductive metabolism of protoplasts.

We found that both the concentrations of 10^6 cells/ml and 10^7 cells/ml provided good performance for tracking cellular metabolism. However, determining the optimal concentration is challenging, as the metabolic activity index (MAI) varies depending on the plant species and growth conditions. The developed system was also tested on different substrates, such as NaCl, which is essential for evaluating protoplast mechanisms of salt tolerance [83] and sensitivity, as well as its role in osmoregulation and nutrient uptake. The system demonstrated effective performance on both plant species tested, revealing significant differences in the metabolic activity of the protoplasts isolated from the two species. These differences likely reflect the distinct phenotypes of the two plants under the tested conditions, confirming, as reported by Gilliard and colleagues, that protoplasts are a valid method

for investigating the perception of both biotic and abiotic stress, as well as different environmental conditions by plants.

Finally, in anticipation of future efforts to develop a high-throughput system for studying various metabolic phenotypes of plant cells, preconfigured Biolog™ Phenotype Microarray plates containing different classes of chemical compounds were selected: 11C, 15B, 18C and 19.

The analysis revealed potential metabolic differences in the sensitivity of protoplasts isolated from two species of the Solanum genus to different concentrations of selected toxic chemical compounds (Supplementary File 1).

It should be noted that Biolog™ has not yet developed PM microplates specifically for plant cells, and current PM microplates are designed for testing the chemical sensitivity of bacterial or fungal cells. Consequently, many substrates included in the plates were not suitable for assessing plant cell metabolic activity and triggered AB dye reactivity not necessarily linked to protoplast metabolism. Therefore, the authors focused on a limited number of compounds from the selected Biolog™ microplates.

Given these constraints, compounds not directly involved in plant metabolism (e.g., antibiotics) were also included. While these may not be physiologically relevant, the lack of a toxic response in protoplasts further supports the reliability of the system.

Importantly, the key aspect in validating our novel application of the Phenotype Microarray platform to plant cells was not the specific nature of the compounds tested, many of which are not typically related to plant metabolic pathways, but rather the ability to detect consistent and distinguishable metabolic responses. This observation demonstrates the potential of the PM approach to be adapted for plant cell phenotyping, offering a new avenue for high-throughput functional analysis.

These findings provide a foundation for developing microplates specifically designed for plant cells, ensuring compatibility with the most suitable dye for these applications.

Conclusions

The Phenotype Microarray (PM) approach described in this study lays the groundwork for future cellular phenotyping investigations aimed at extrapolating whole-plant metabolic responses from cellular-level activity. This method holds significant potential for enabling comparative metabolic analyses among different plant cultivars, species, or genetically modified lines, particularly under environmental stress conditions such as exposure to elevated levels of heavy metals, toxic compounds, or salinity. As such, the PM platform may represent a powerful tool for elucidating plant adaptive responses through high-resolution cellular profiling. An interesting aspect of using PM to study the metabolic phenotypes of plant cells is its potential application in systems biology studies on plant cell reprogramming. Indeed, the real-time monitoring capabilities of the PM platform could be effectively integrated with time-course analyses of protoplast reprogramming, providing a complementary approach to *-omics* investigations, including epigenetic modifications and gene expression profiling. Nevertheless, while protoplast studies offer valuable insights, the absence of the cell wall and the isolation process may influence certain cellular behaviors. Therefore, validating findings in whole plants will be essential to confirm PM predictions.

Abbreviations

PM	Phenotypic microarray
MAV	Metabolic activity value
AD	Activity Delta
AB	Alamar blue

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13007-025-01378-5.

Additional file 1. List of chemical compounds contained in PM11C, PM15B, PM18C and PM19. The number of plates, their location of compounds, their related mode of actions and doses are reported together with others information. In bold the chemical used in the sensitivity analysis

Additional file 2

Additional file 3

Additional file 4. Figure S1. Percentage Variation of AB004 dye' consumption of protoplast at different concentration. % V was calculated among initial and finalvalues of the kinetic curves detected by the OmniLog software. Data are expressed as average. Figure S2. Activity delta of *S. tuberosum* and *S. lycopersicum* protoplastson different NaCl concentrations, compared to the control. The letters above the bars indicate statistically significant differences between groups, calculated using ANOVA followed by a Tukey post-hoc test. Activity delta was calculated as the ratio between the AUCat a specific NaCl concentration and the AUC in the absence of NaCland was determined by AB004 consumption. Table S1. Statistical differences in persistence of 10^6 and 10^7 protoplasts/mL of *S. lycopersicum* and *S. tuberosum* at different concentrations of NaCl at incubation time of 25, 50, 75 and 92 hours. Mean Metabolic Activity Index are reported for each condition; different superscript letters indicate difference different letters indicate statistical differences based on the Tukey test or Dunn test. Table S2. Percentage variationof metabolic activity in presence of the selected toxic compounds present in Biolog microplates. %V was calculated among initialand finalvalues for each substrate tested in the Biolog™ microplates 11C, 15B, 18C and 19. The second column indicates the increasing concentration of the toxic compound, from lower [1] to higher [4] concentration. *Note: Biolog™ does not disclose the exact concentrations of the tested compounds.*

Author contributions

AC, AM, CV, DP and FD conceived and designed the study. FD, GA and AC performed protoplasts isolation and PM experiments. RN, SB and GA performed in vitro plant cultivation. AC, GA and AB analyzed the data and

validated the study. AC prepared the draft with the help of GA and FD. All authors contributed and approved the final manuscript.

Funding
The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 12 March 2025 Accepted: 22 April 2025
Published online: 07 May 2025

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