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Beneficial effects of plant growth-promoting yeasts (PGPYs) on the early stage of growth of zucchini plants

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ABSTRACT

The use of microbes capable of beneficially interacting with plants is essential for advancing climate-smart agriculture. This approach aims to reduce chemical use while simultaneously enhancing crop productivity. This implies efforts to optimize the criteria for selecting potential plant growth promoters (PGPs), focusing also on yeasts, only recently investigated for their PGP potential. The present study employed a set of Ascomycetes and Basidiomycetes yeasts to test their PGP properties on zucchini (*Cucurbita pepo* L.), chosen as a fast-growing plant with a vast economical interest. Yeasts were tested alone and as consortium. Seed inoculation with yeasts boosted the early phase of growth of the zucchini plants, primarily affecting the root development. Three strains belonging to the species *Schwanniomyces etchellsii, Zygotorulaspora florentina* and *Holtermanniella festucosa* induced a strong and significant enhancement of weight and length of both epi- and hypogeal parts of the plant. Furthermore, the presence of yeasts induced strain-specific modulations in the biochemical profiles of soil, primarily detected in the rhizosphere. This suggests an active interaction between the roots and the inoculated yeast cultures.

1. Introduction

Plant growth requires large amounts of fertilizers, especially in intensive horticulture, leading to chemicals dispersal in the environment and sometimes also in the water bodies. Moreover, the growing demand for foodstuffs increases the request for more productive crops leading to greater use of fertilizers and chemicals [1,2]. In this context, the boost given by beneficial microbes is an important tool to optimize the use of fertilizers, decrease the usage and loss of chemicals in the environment and enforce the principles of climate-smart agriculture [3,4]. It is widely demonstrated that rhizosphere microbial communities significantly influence plants and other environmental members [4]. Managing this system requires selecting potential plant growth promoters (PGPs) and optimizing their use under the complex rhizospheric conditions. In this environment, the selected PGPs will interact with soil, plants and other microorganisms, whether seed-borne or already present in the soil. Given the complexity of these interactions the research should focus on the specific combinations of plant, ecological conditions and potential PGPs. Despite the recognized bacterial role in promoting plant growth, recent research on plant growth promoting yeasts (PGPYs) suggest that these microorganisms are also promising candidates for sustainable agricultural practices, adhering to environmental and food safety standards [5]. PGPYs are Generally Recognized as Safe (GRAS), further emphasizing their potential in agricultural applications [5]. PGPYs belong mainly to Ascomycetes phylum with most of species belonging to the genera *Saccharomyces*, *Candida, Rhodotorula* and *Cryptococcus* [5]. They are primarily isolated from the rhizosphere where they can act to promote and support plant growth through different mechanisms. One of their main activities is linked to the phytohormones production, crucial in promoting plant growth and resistance/tolerance to abiotic stresses as well as in modifying the root architecture by increasing the uptake of nutrients and water [6]. PGPYs can also increase the

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bioavailability of nutrients by secreting acids, enzymes and chelators such as siderophores and contribute to the solubilization of phosphate necessary for plant growth [5,7]. The search for yeasts to be employed for a sustainable agriculture is also enforced by their potential as biocontrol agents of pathogenic fungi, being GRAS for field applications. Many yeast species, in fact, can compete with filamentous fungi by growing faster than the latter, colonizing different parts of plants or by producing antifungal compounds [8]. Furthermore, some strains are known to release volatile organic compounds (VOCs) involved in the plant resistance to disease and in its tolerance to abiotic stress [9]. Finally, enhancing crop productivity involves formulating yeast consortia capable of maximizing the PGP traits across various strains, thereby minimizing reliance on chemical fertilizers [10–12]. In this framework, the present study aims at testing the PGP properties of ten different yeast species, alone and as consortium, in supporting the growth of zucchini plants under controlled conditions. Summer squash (*Cucurbita pepo* L. var. "Nano verde di Milano") has been chosen as fast growing almost ubiquitous plant with a huge economical interest and with problems to balance production and environmental sustainability [13–16].

2. Materials and methods

2.1. Plant and soil

Zucchini seeds (*Cucurbita pepo*, cv. "Nano verde di Milano") were provided by Bavicchi S.p.a. and cultivated on "VULCAMIX" commercial soil [17,18]. Soil electrical conductivity (EC) was measured from 1:5 (w v^{-1}) soil-water extract [19]. $EC_{1:5}$ of supernatant was measured by conductivity EC-meter CRISON-GLP-31 at 25.0 ±0.1 °C [20]. Final value was converted to standard EC_e parameter, by using the conversion factor proposed by Lee, Hong *et al.* [21]. Other analytical parameters measured were: soil extract 1:5 (w v^{-1}) pH with pH-meter and soil moisture % by thermo-balance [22–24] (Table 1).

2.2. Yeast cell cultures

The ten yeasts strains used in the study were provided by the CEMIN Microbial Collection (CMC) of the Microbial Genetics and Phylogenesis Laboratory of CEMIN (Centre of Excellence on Nanostructured Innovative Materials for Chemicals, Physical and Biomedical Applications – University of Perugia). The species were selected by including both Ascomycetes and Basidiomycetes to investigate their relative effectiveness. This selection encompassed species typically isolated from soil as well as those from other sources. The aim was to determine whether positive effects could be achieved with yeasts not strictly associated with soil (Table 2).

Each strain was grown on YEPD (Yeast Extract 10 g L^{-1}, Peptone 10 g L^{-1}, Dextrose 20 g L^{-1}) medium at 25°C under shaking at 120 rpm. After 18 h, each pre-inoculum was inoculated at $OD_{600\ nm}$= 0.1 in 50 mL YEPD (Yeast Extract 10 g L^{-1}, Peptone 10 g L^{-1}, Dextrose 20 g L^{-1}) and incubated in the same growth conditions [25]. The final inoculum was prepared by calibrating the $OD_{600\ nm}$ to the final concentration of 2 × 10^8 cell mL^{-1}.

Table 1
Soil and irrigation water parameters.

Soil - VULCAMIX	
$EC_{1:5}$	0.05 dS m^{-1}
EC_e	0.3 dS m^{-1}
Moisture	20.0%
Granulometry	0–3 mm
pH	8.12
Irrigation water	
EC_w	0.9 dS m^{-1}
pH	7.6

2.3. In vitro screening of yeasts plant growth promoting traits

Yeasts were grown overnight in 5 mL YEPD (Yeast Extract 10 g L^{-1}, Peptone 10 g L^{-1}, Dextrose 20 g L^{-1}) under shaking (150 rpm) at 30 °C. Each culture was then collected by centrifugation (3500 rpm, 10 min, 4 °C) and washed three times with 0.9 % sterile saline solution. Cell density was standardized using a McFarland barium sulfate standard 1, corresponding to 1 × 10^7 CFU mL^{-1}, by adding sterile 0.9 % saline solution. To test yeasts as consortium, 1 × 10^5 CFU mL^{-1} of each strain were inoculated in the proper medium. All tests were performed in triplicate under shaking (150 rpm) at 30°C.

2.3.1. Phosphate solubilization

Phosphate solubilization was evaluated in broth medium. Five µL of standardized yeast suspension were inoculated in 5 mL of NBRIP broth (Glucose 10 g L^{-1}, $Ca_3(PO_4)_2$ 5 g L^{-1}, $MgCl_2$ 6 H_2O 5 g L^{-1}, $MgSO_4$ 7 H_2O 0.25 g L^{-1}, KCl 0.2 g L^{-1}, $(NH_4)_2SO_4$ 0.1 g L^{-1}) supplemented with 0.025 mg mL^{-1} of bromophenol blue (BPB), designated as NBRIP-BPB, and incubated for 7 days. $OD_{600\ nm}$ was taken by using UV/visible spectrophotometer [26]. Moreover, 5 µl of standardized yeast suspension was spot inoculated on NBRIP medium with agar, and incubated at 30 °C. After 7 days of incubation plates were observed for development of a clear halo zone around the colony, the halo's diameter was evaluated according to Ambrosini and Passaglia [27]. Results were expressed as percentage difference of absorbance, respect to not inoculated control.

2.3.2. Indole acetic acid production

The phytohormone indole acetic acid (IAA) production was estimated using the Salkowski reagent (1 mL of 0.5 M $FeCl_3$ in 50 mL of 35 % $HClO_4$) following the protocol proposed by Sun et al. [28]. Five µL of standardized yeast suspension were inoculated in YEPD (Yeast Extract 10 g L^{-1}, Peptone 10 g L^{-1}, Dextrose 20 g L^{-1}) supplemented or not with the precursor L-Tryptophan (0.1 %) (Sigma-Aldrich, St. Louis (MO), USA) and incubated under shaking (150 rpm) at 30 °C. After the incubation, the cultures were centrifuged at 4 °C for 10 min (10.000 rpm). The supernatant was mixed with Salkowski (1:2 v:v) and incubated in the dark for 1 h. Development of red color indicated the presence of IAA. $OD_{600\ nm}$ was monitored at 540 nm by using UV/visible spectrophotometer. Standard curve of IAA was used to measure the concentration of IAA produced (Supplementary table S7).

2.3.3. Siderophore production

The strains were also quantitatively assessed for siderophores production using CAS (Chrome Azurol Sulfonate) reagent [29]. Isolates were inoculated in siderophore-inducing medium (SIM) and incubated for 7 days. Yeast cultures were then centrifuged (10.000 rpm, 10 min) and each supernatant was mixed with the CAS solution (1:1 v:v). After 1 h of incubation at room temperature absorbance 630 nm is measured to estimate the loss of blue color to orange/yellow. Resulting values were reported as relative difference of absorbance, compared to not inoculated control.

2.4. Mesocosm experimental setup

The effect of yeast inoculation on zucchini growth was investigated in a mesocosm experiment carried out in a greenhouse equipped with automatic control of temperature (25 ±5.0 °C) at the Bavicchi S.p.a company (43.09852283829231°N– 12.452456473876751°W, Perugia, Italy). The ten selected yeasts and the microbial consortium of the 10 species (Table 2) were tested with three-fold replications. For every thesis, 1-L pots (13 cm diameter) were filled with 1 kg of VULCAMIX soil. For each pot, three zucchini seeds were inoculated with 2 mL of yeast suspension in culture medium, to provide some organic matter to support the yeast growth before the seed germination. The control pots were prepared in triplicates, without the addition of yeast inoculum on

Table 2
Yeast species used as inoculum.

CEMIN code	Species	Abbreviation	Phylum	Isolation source
CMC 1661	*Holtermanniella festucosa*	*Hf*	Basidiomycetes	Soil
CMC 1693	*Debaryomyces hansenii*	*Dh*	Ascomycetes	Soil
CMC 322	*Debaryomyces prosopidis*	*Dp*	Ascomycetes	*D. carbonaria*
CMC 836	*Debaryomyces udenii*	*Du*	Ascomycetes	Soil
CMC 1643	*Naganishia uzbekistanensis*	*Nu*	Ascomycetes	Soil
CMC 1688	*Papiliotrema terrestris*	*Pt*	Ascomycetes	Soil
CMC 812	*Schwanniomyces etchellsii*	*Se*	Ascomycetes	Pickle fermentations
CMC 1669	*Solicoccozyma phenolica*	*Sp*	Basidiomycetes	Soil
CMC 957	*Wickerhamomyces anomalus*	*Wa*	Ascomycetes	Oil
CMC 1657	*Zygotorulaspora florentina*	*Zf*	Ascomycetes	Grape must
-	Microbial consortium	Consortium of the 10 species	-	-

seeds. VULCAMIX soil sterilization was avoided to best mimic the real greenhouse conditions, where the soil sterilization process is not practicable. Pots were then placed in shallow tanks, positioned on a cart and watered every 3–5 days to maintain soil humidity around 20%. Soil samples were collected at 12, 21 and 33 days from planting, corresponding to seedling emergence, half plant growth and advanced plant growth, respectively. Rhizosphere soil samples were collected at 33 days of growth, when plants were eradicated, stored in plastic bags and transported to the laboratory for further analyses.

2.5. Plant analyses

For each thesis, three plant replicates were randomly sampled and cut at the collar level, to separate epigeal and hypogeal portion. Then, respective lengths were measured. Fresh and dry plant/roots weights were determined after drying at 70°C for 48 h [30] (Supplementary table S1). Total chlorophyll and carotenoids content was evaluated applying the protocol extraction and equation proposed by Siebeneichler et *al.* [31]. Final values were expressed in mg g^{-1} [32] (Supplementary table S1).

2.6. Assessment of fungal cell density in the mesocosm experiment

Soil samples were randomly collected from each pot after 12, 21 and 33 days of zucchini growth. Samples at 12 and 21 days were picked up with sterile tweezers close to each plant while, at 33 days, plants were eradicated by separately collecting the soil adhered to roots (rhizosphere soil) and the residual soils. All soil samples from the replica pots of the same thesis were collected in bulk in falcon tube and stored at 4°C until they were analysed.

Fungal cell density (CFU g^{-1}) was determined by viable cell count (Supplementary table S1). For each thesis, 1:5 (w v^{-1}) soil suspension was prepared by adding 5 mL of sterile physiological solution (0.9 % NaCl) to 1 g of fresh soil. Soil suspensions were then serially diluted and 100 µL were plated in triplicate onto YEPD agar medium, supplemented with Chloramphenicol (0.5 g L^{-1}) and Rose Bengal (0.005 g L^{-1}). Plates were incubated for 48 h at 25 °C. For each sampling time, the ratio between the cell density of the rhizosphere and that of the soil samples was calculated and expressed as Rhizosphere *vs* Soil Ratio (RSR).

2.7. Biochemical profiles of soil

FT-IR analysis was employed to evaluate the influence of yeast addition on the biochemical profiles of soil. From each sample, 2 g of soil were suspended in 10 mL of HPLC grade water [1:5 (w v^{-1})], vortexed for 10 min and left to settle for 10 min. For each sample, 35 µL volume of supernatant was sampled for three independent FT-IR readings (35 µL each, according to the technique suggested by Essendoubi and colleagues [33]. FT-IR analysis was carried out with a TENSOR 27 FT-IR spectrometer, with HTS-XT accessory (BRUKER Optics GmbH, Ettlingen, Germany). The measurements were performed in transmission mode. All spectra were recorded in the range between 3400 and 700 cm^{-1}. Spectral resolution was set at 4 cm^{-1}, sampling 256 scans per sample to obtain high quality spectra. The software OPUS v. 6.5 (BRUKER Optics GmbH, Ettlingen, Germany) was used to assess the quality test, subtract the interference of atmospheric CO_2 and water vapor, correct the baseline (rubber band method with 64 points), apply vector normalization to the whole spectra and calculate the average spectra from three replicates from each sample [34] (Supplementary table S2).

2.8. Statistical analyses

Statistical analyses were performed in Microsoft Excel and R v4.2.2, using packages "*devtools*", "*tidyverse*", "*factoextra*". Pakcgaes "*ggbiplot*" and "*ggfortify*" were used to plot the results [35].

2.8.1. PGP traits

Analysis of Variance (ANOVA) was performed on each PGP trait dataset, to assess the statistically significance among all yeasts. One-tail paired T-test (MS excel) was performed to compare IAA value in presence or not of Try (Supplementary table S3).

2.8.2. Growth parameters

One-tail paired T-test (MS excel) was used to compare each inoculum with the control in terms of both cellular density and plant growth parameters (Supplementary tables S4, S5, S6). Statistical significance was determined considering p values smaller than 0.05 (*) and 0.001 (**). Principal Component Analysis (R environment) was performed on plant growth data to characterize the influence of all inocula on the hypogeal and epigeal fractions of the plant, considering fresh weight, dry weight and length. The same analysis was performed for the pigments content.

2.8.3. FT-IR spectral analysis

FT-IR spectra were pre-processed using OPUS 6.5 software by baseline and vector normalization. The normalized spectra were exported as. txt for further analysis in R environment. Mean spectra were calculated from three independent technical replicates and Significant Wavelengths Analysis (SWA) was performed on five different FT-IR spectral regions (Table 3) to compare data obtained from bulk or rhizosphere soil and control [36]. Briefly, SWA analysis was performed on mean spectra from each sample with Test-t ($p < 0.01$), wavelength by wavelength for each spectral region.

3. Results and discussion

3.1. Plant growth-promoting traits

The screening of the PGP traits was performed on the ten yeasts alone and as consortium under dynamic conditions at 30 ºC (Table 4).

Table 3
Characteristic spectral regions of soil FT-IR spectra and respective assigned components. Peaks assignment was carried out according to [37–40].

Regions	Peaks (cm^{-1})	Range (cm^{-1})	Components assignment	
			Microbial cells	Organic soil
			Functional group	**Matter**
s1	3623	3625–3615	/	O_2 - containing organic matter
s2	1637	1650–1633	Amides (1800–1500)	Amides Ketones Quinones Lignin / Carboxyls Hydrophilic materials of SOM
s3	1041	1060–1010	Carbohydrates (1200–900)	Polysaccharides groups
s4	914	945–870	Phosphorylated proteins DNA/RNA Nucleotides $CaCO_3$ minerals	Benzoic acid Pyranose ring cellulose
s5	650	871–600 (~650)	Phenylalanine Tyrosine Tryptophan Nucleotides	/

3.1.1. Phosphate solubilization

All yeasts tested exhibited *in vitro* phosphate solubilising ability, with values ranging from 2.07 ±0.15 %, recorded for *W. anomalous*, to 28.45 ±1.74 %, for *Z. florentina*. Among the others, only *S. phenolica*, *P. terrestris*, *N. uzbekistanensis*, *S. etchelsii* and the consortium showed values above the average detected for these yeasts (14.64). It is known that phosphorus solubilizing microorganisms play a key role in phosphorus nutrition by improving its availability for plants through solubilization and mineralization processes [41,42]. In fact, a considerable portion of the soil phosphorous exists in the insoluble form, which is not readily available to plants. Moreover, substantial amounts of chemical fertilizers are applied to address phosphorous deficiency, thereby escalating both the expenses of crop cultivation and environmental repercussions. The selection of novel yeasts capable of solubilizing inorganic phosphorus, promoting plant growth, and reducing chemical usage represents an intriguing strategy for sustainably managing phosphorus deficiency in agricultural soils [43].

3.1.2. Production of biomolecules

However, a preliminary test conducted under different conditions (static, 25°C) demonstrated that some of these yeasts possess this ability. This suggests that the presented results could be attributable to the testing platform rather than a lack of this ability in the yeasts evaluated. This consideration underlines the need to optimize and standardize the analytical methods and to develop specific protocols for the analysis of the PGP traits of yeast [44]. IAA is an important phytohormone used by plants to regulate growth [45]. It is known that inoculation with IAA-producing yeasts enhances root growth resulting in increasing root surface area and root hair formation, promoting the nutrition of plants [28,46]. The current literature indicates that IAA synthesis is indeed a frequent characteristic among yeasts [28,46,47], which can use different producing pathways influenced by the presence or absence of tryptophan [46,48]. As such, this study specifically tested the IAA-producing ability of the yeast strains under study with or without biochemical precursor L-Tryptophan, to compare the Try-dependent or independent pathway of synthesis. Despite all yeasts were able to produce IAA in the absence of exogenous tryptophan, their capacity increased several-fold in the presence of L-Tryptophan (0.1%) with statistical significance, excepted for *H. festucosa* ($p < 0.01$). Interestingly, the maximum amount of IAA was detected for the yeast consortium in both tested conditions (10 ±0.60 vs 58.80 ±11.40 μg mL^{-1}). Among the single cultures, the best producers were *W. anomalus* (27.64 ±2.55 μg mL^{-1}), *P. terrestris* (19.64 ±3.47 μg mL^{-1}) and *D. udenii* (18.07 ±0.2 μg mL^{-1}). Overall, these results indicated that the ability to produce IAA of these yeasts varies significantly between species according to a Try-dependent pathway, confirming what has already been reported by other authors for PGPYs [28,46,49].

3.2. Dynamic of yeast cell density over the zucchini growth

Yeast cell density decreased from 3 to 2 log (CFU g^{-1}) during the 33 days of observation (Fig. 1a). At 12 days from the inoculum, cell densities ranged from 5 to 6 log (CFU g^{-1}), with an average of 1.7 $\times 10^6$ CFU g^{-1} and a significant variability among strains. Some strains were able to increase the total count at 21 days, whereas other maintained the density recorded at 12 days or showed some decrease. Finally, counts in all treatments decreased at 33 days, in some cases reaching a density similar to that of the control, whereas other theses (*D. prosopidis, D. udenii, P. terrestris, S. etchellsii, S. phenolica* and the consortium) showed densities around 4–5 log (CFU g^{-1}), *i.e.* 1–2 orders of magnitude over the control. The rhizosphere soil could be sampled only at the end of the test, *i.e.,* at 33 days, when plants were eradicated. Rhizosphere soil at 33 days showed yeast cell densities ranging from 3 log (CFU g^{-1}) in the case of *W. anomalus* treatment, to 6 log (CFU g^{-1}) with an average 1.62 $\times 10^6$ (CFU g^{-1}), very close to that of the soil at 12 and 21 days (Fig. 1b).

Interestingly, the most successful cultures that ranged over 4 log

Table 4
Ability of yeasts to solubilize phosphate and produce IAA and siderophores.

CEMIN code	Species	P-solubilization (%)	IAA no Trp (μg mL^{-1})	IAA Trp (μg mL^{-1})	Siderophore (%)
CMC 1693	*Dh*	8.15 (±1.97)	5.58 (±0.98)	14.22 (±0.2)	-
CMC 322	*Dp*	4.14 (±1.14)	3.84 (±0.36)	14.07 (±0.38)	-
CMC 836	*Du*	10.95 (±1.3)	5.6 (±1.56)	18.07 (±0.2)	-
CMC 1661	*Hf*	5.16 (±0.47)	0.28 (±0.39)	5.41 (±2.62)	-
CMC 1643	*Nu*	21.24 (±0.87) *	2.96 (±0.14)	15.41 (±0.22)	-
CMC 1688	*Pt*	26.31 (±6.77) *	4.48 (±0.08)	19.64 (±3.47	-
CMC 812	*Se*	24.19 (±0.6) *	3.3 (±0.31)	12.26 (±0.76)	-
CMC 1669	*Sp*	15.8 (±1.53) *	0.9 (±0.29)	6.21 (±0.92)	-
CMC 957	*Wa*	2.07 (±0.15)	5.98 (±0.49)	27.64 (±2.55)	-
CMC 1657	*Zf*	28.45 (±1.74) *	1.03 (±0.67)	11.18 (±1.5)	-
-	Consortium	26.0 (±5.10)	10.0 (±0.600)	58.80 (±11.40)	-

Legend. Values report the means of three replicates (± SD). **P**hosphate (P) solubilising ability and siderophore production are reported in percentage, as relative difference in absorbance compared to the not inoculated control. The production of IAA was evaluated in YEPD supplemented (IAA + Try) or not with the precursor L-Tryptophan (IAA no Try). Negative controls (i.e. not inoculated tubes) were incubated 7 days at the same conditions of the samples. For IAA production, they showed an average absorbance of 0.20 for YEPD not supplemented with Try and 0.21 for YEPD+Try, which correspond to 0 μg mL^{-1} IAA and 3 μg mL^{-1} IAA respectively. All data are significantly different from the Consortium ($p < 0.001$), excepted for those indicated with *. Species were named according to Table 2.

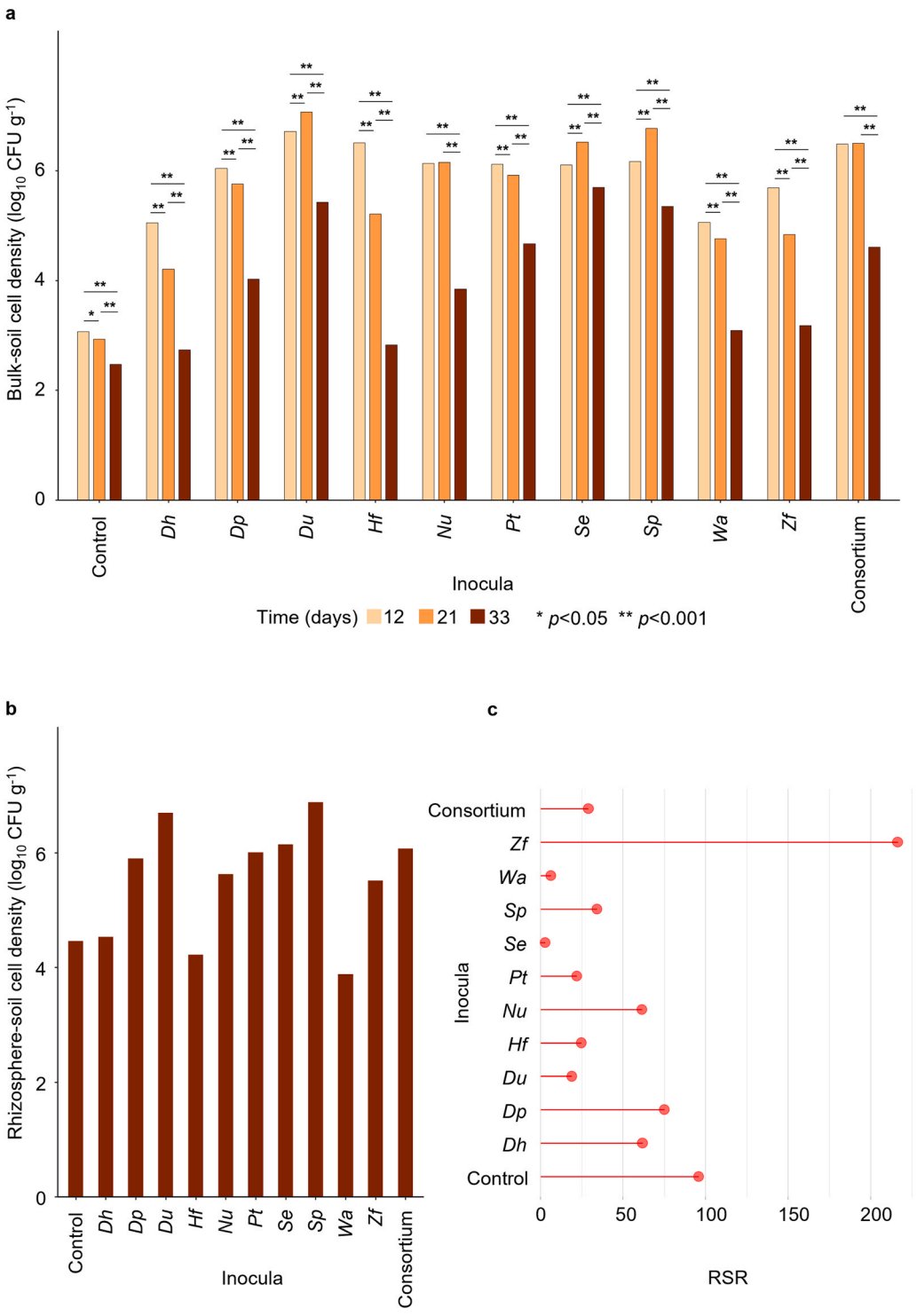

Fig. 1. Evolution of cell density of the total yeasts in soil and rhizosphere samples over time. Cell densities were reported as $\log_{10}$ CFU g^{-1} (n=3). (a) Evolution of yeast cell density in soil samples collected at 12, 21 and 33 days of zucchini growth. For each thesis, data were compared in pairs with the t-Test analysis and statistical significance was reported accordingly as * ($p < 0.05$) and ** ($p < 0.001$); (b) Evolution of yeast cell density in rhizosphere soil samples collected after 33 days from the inoculum (plant eradication). (c) Rhizosphere *vs* soil cell density ratios (RSR) values at 33 days of zucchini growth. Yeast species were indicated according to the abbreviation listed in Table 2.

(CFU g^{-1}) in soil samples, were the same that varied over 5 log (CFU g^{-1}) in the rhizosphere at the same time. All inocula resulted in a higher cell density in proximity of the rhizosphere than in the soil (Fig. 1a, b), with rhizosphere *vs* soil cell density ratios (RSR) ranging from 6 to 216 (Fig. 1c). RSR values suggest that some cultures were more able than other to take advantage by the presence of root exudates, seemingly more present in the rhizosphere than in the rest of the soil in the pot. *W. anomalus* and *S. etchellsii* showed RSRs of 6 and 3 respectively, below the average of the whole test (RSR=50), while four strains were over the average: *D. hansenii* (RSR=62), *D. prosopidis* (RSR=75), *N. uzbekistanensis* (RSR=61) and *Z. florentina* (RSR=216). Some strains showed high cell density in both soil and rhizosphere throughout the

mesocom experiment, *N. uzbekistanensis,* Z. *florentina* and *D. prosopidis* had around 3 log (CFU g^{-1}) in soil at 33 days, suggesting that these species are particularly stimulated by the presence of root exudates.

An important question raised by this study is why cell densities declined after 12 days in soil relatively far from the roots. One plausible explanation is the gradient of organic matter from the roots to the inoculation site, which also explains why cells were more prevalent in the rhizosphere than in the surrounding soil at the end of the experiment [50]. Additionally, the intentional omission of organic matter to avoid introducing further variables might have had a negative selective effect on some species, as well as created nutritional competition between the plant and the additional microbiota [51,52]. This aspect is of paramount importance for the final application of plant growth promoters in agriculture. Indeed, it is difficult to envision adding expensive, sugar-rich substrates, as complex organic matter would only become usable after a lengthy degradation process involving the resident soil microbiota, which would be too time-consuming to support the rapid growth of yeasts. These considerations highlight the need to develop relatively inexpensive substrates that are readily usable by the inoculum well before root exudates become significantly available, which only occurs once plants are actively photosynthesizing. Finally, our data do not allow us to exclude that yeast cells exerted a beneficial effect after their autolysis, by releasing the cytoplasm which would be both a source of nutrients and bio stimulant agents for the plant. These considerations indicate the need to develop relatively inexpensive substrates readily usable by the inoculum, well before roots exudates, not significantly available before plants being actively photosynthesizing [53,54]

3.3. Modulation of plant growth induced by yeast addition

Overall, the inoculation of the seeds with yeasts produced a general positive effect on the early phase of growth of the zucchini plants for all the tested parameters, *i.e.,* fresh and dry weight, epi- and hypogeal growth and the production of pigments (Fig. 2).

3.3.1. Effect on epigeal and hypogeal growth

Only *Z. florentina*, *S. etchellsii*, and *H. festucosa* simultaneously improved both epigeal and hypogeal growth, despite not being among the yeasts that exhibited the most growth during the experiment, either in the soil or the rhizosphere. (Fig. 1, Fig. 2a and c). The only species with a significant activity on plant elongation was *D. udenii* ($p < 0.05$). On the contrary, *S. phenolica, D. prosopidis* and *D. hansenii* did not significantly promote the growth of zucchini plants while the addition of *P. terrestris* and *W. anomalus* induced a significant decrease ($p < 0.05$). The PCA analysis (Fig. 2b, d), displaying more than 99 % of the whole variability, supported the statistical analyses and showed that the vectors of dry and fresh weight diverged significantly from that of the plant length. Interestingly, *Z. florentina* and *S. etchellsii* were also those strains that exhibited the higher *in vitro* phosphate solubilising ability. On the contrary, the activity of *H. festucosa* was non supported by the PGP traits detected *in vitro* (Table 4).

Overall, the effect of the yeast inoculation was more effective on roots than on the aerial portion of the plant, as shown by the scale of the percentual variations (Fig. 2c). Root length was never affected by the presence of yeasts, except by *W. anomalus* and *P. terrestris* which produced a significant depression of all tested parameters. On the other hand, a significant improvement of the weights was induced, increasingly, by *H. festucosa, S. etchellsii, Z. florentina* and *N. uzbekistanensis,* that enhanced the root weight by ca. 300%. The effect exerted by *N. uzbekistanensis* on the root weight was supported by the *in vitro* tests, being the only strain that coupled high values for both P-solubilization and IAA production with a Tryptophan-dependent pathway (Table 4). Once again, the PCA confirmed these findings and showed that independence of the length from the two weight descriptors that are almost on the same projection of the two components.

Taken together, these data confirm that the attribute of "PGP" is primarily linked to the effects exerted by the complex *in vivo* interaction between microorganism, plant, and environment, rather than the activities recorded *in vitro*. This finding paves the way for more in-depth investigations to optimize the analytical settings for these studies [12,44,55]. Furthermore, among the three species that promoted both epi- and hypogeal growth of zucchini plants, *S. etchellsii* and *Z. florentina* are Ascomycetes while *H. festucosa* is a Basidiomycete. Whereas the latter is usually isolated from soil, *S. etchellsii* and *Z. florentina* were introduced in this study to test whether positive effects could be obtained with yeasts non strictly related to soil [56]. *S. etchellsii* was usually found in salty environments as brines while *Z. florentina* is isolated very often from sugary juices and substrates and very little is known about their ability to positively interact with plants [5,57,58]. To our knowledge, these results are the first to show the PGP ability of these three yeast species, opening the discussion on the criteria for the selection of PGP yeasts. Most articles report that PGPYs mainly belong to the phylum Ascomycetes and that most of them are isolated from the rhizosphere [59]. The fact that *S. etchellsii* and *Z. florentina* have not been isolated from soil suggests the potentially more beneficial yeast-plant interactions beyond those identified through direct isolation. This highlights a significant opportunity for exploring the biotechnological potential of numerous available yeasts, extending beyond those traditionally considered due to their association with the soil environment.

3.3.2. Modulation of pigments

The presence of different yeasts in the rhizosphere induced the zucchini plants to differently produce pigments (Fig. 2e, f). Among the three species with good performances in the induction of plant growth, *H. festucosa* had no effects on the pigments, while both *S. etchellsii* and *Z. florentina* prompted a significant improvement of total chlorophyll ($p < 0.01$). This observation is consistent with the evidence that the last two yeasts induced more epigeal growth than any other yeast strains and roughly twice as much as *H. festucosa* (Fig. 2a). The *W. anomalus* and *D. udenii* strains induced a significant increase of both carotenoids and total chlorophyll ($p < 0.05$) (Fig. 2e).

3.3.3. Effect of yeast consortium on Zucchini growth

One hypothesis of this paper was to evaluate the effectiveness of the consortium compared to single yeast cultures. When tested *in vitro*, the consortium significantly reached the highest values for all the screened activity (Table 4). On the contrary, the results of the plant tests showed that it performed averagely in most cases (Fig. 2). Indeed, the sole significant *in vivo* activity observed was the increase in fresh and dry root weights ($p < 0.05$) (Fig. 2c). This behavior could be explained by two alternative hypotheses. Firstly, the consortium contained reduced amounts of each culture, as the cultures were calibrated to the same total cell density. Consequently, each species was present in the mixed culture at approximately 11 % of the cell density compared to pure cultures. Moreover, it is plausible that competition among the yeasts in the consortium restricted the growth of many components [60]. An alternative hypothesis suggests that the consortium produced metabolites with both positive and negative effects on zucchini plants, resulting in an “averaged” phenotypic trait as a balance of the effects of the single cultures [61]. This view is supported by the observation that some yeasts had inhibitory effects on both epigeal and hypogeal growth (Fig. 2a and c). Further analyses are underway to elucidate this issue, employing techniques capable of quantitatively defining the growth of each single member of the consortium. Given the widespread use of consortia in microbial biotechnologies applied to agriculture, understanding the mechanisms governing them is of significant interest [11,12,62,63].

3.4. Variation of the whole soil metabolome induced by yeast addition

The aforementioned data give some hints on the possible mechanisms governing the yeast-plant interactions, that are more likely influenced by a complex network of molecules produced by both yeast

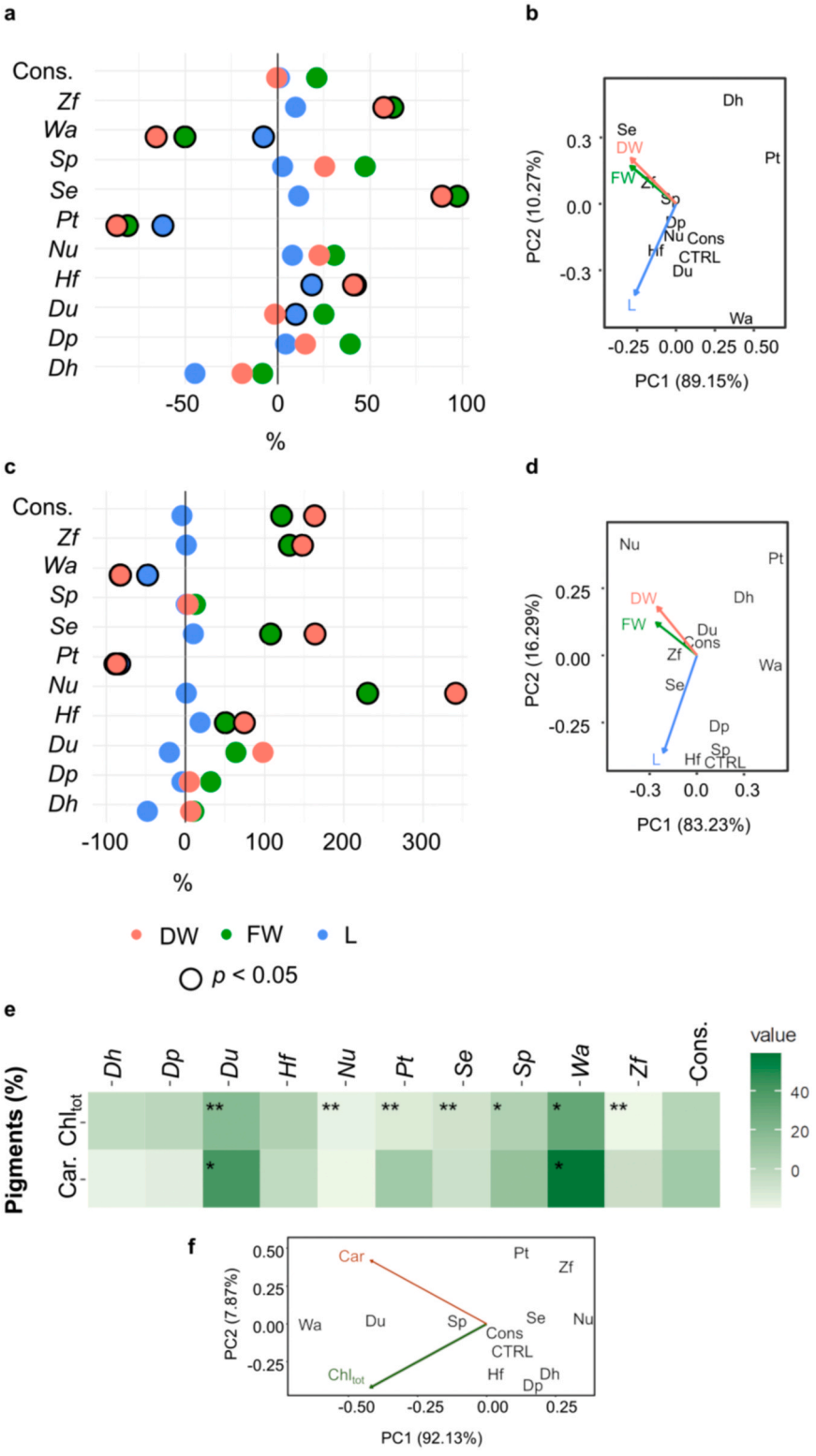

Fig. 2. Differences imparted by the inoculum of yeasts on the plant parameters at 33 days of zucchini growth. Differences in length (L), fresh weight (FW) and dry weight (DW) were separately reported for the epigeal (a) and hypogeal (c) part of the plant as percentage difference (%) respect to the control (n=3). Data statistical significance was indicated by black-delimited circles ($p < 0.05$). (e) Percentage differences in leaf pigments content (mg g^{-1}) between control and inoculated plants (n=3). The significance between controls and tests was reported as * ($p < 0.05$) and ** ($p < 0.01$). Principal Component Analysis of descriptors for epigeal (b), hypogeal (d) part of the plant and pigments content (f). Yeast species adopted as inoculum were indicated according to the abbreviation listed in Table 2.

strains and plant. To gain insight on this aspect, FT-IR biochemical profiles of soil extract samples were analysed (Fig. 3).

Overall, the addition of yeasts induces a significant alteration in the metabolomic profile of the soil ($p < 0.01$), exhibiting changes that are predominantly culture-specific and time and space dependent (soil *vs* rhizosphere). Yeasts addition induced differences in five spectral regions, denoted as s1 to s5, as detailed in Table 3. Analysis of soil profiles highlighted how yeasts growth dynamics was influenced by root proximity, confirming what already discussed for the evolution of cell densities during the test (Fig. 1). Most notable differences were observed in the s4 and s5 regions, mainly attributable to nucleotides and amino acids (Table 3) and followed the trend of cell density.

Far from the rhizosphere, the maximum number of significantly altered wavelengths occurs at 12 days, subsequently declining until the end of the test. *D. hansenii* and *D. prosopidis* inoculation produced strong alterations at 12 days, coinciding with their peak cell density (CFU g^{-1}). The same pattern was shown by *D. udenii*, in the s4 region, after 21 days of zucchini growth (Fig. 1a, Fig. 3b). The prevalence of metabolomic alterations in the early stages of the test, when yeast concentration in bulk soil is maximal, supports the evidence that these metabolomic changes are attributable to yeast metabolism. Similarly, significant alterations in the rhizosphere at the end of the experiment could be explained by the higher yeast cells concentration close to the roots. This finding suggests an active interplay between the roots and the added cultures, although the question on whether these metabolites derive from the plant stimulated by yeasts or directly by yeasts requires further analyses [64–67].

4. Conclusion

A sustainable alternative to the use of synthetic fertilizers and pesticides in agriculture is the use of plant growth promoting microbes (PGPM), with yeasts emerging as promising candidates due to their Generally Recognized as Safe (GRAS) status. In this study, ten yeast strains were tested for their PGP potential, both alone and as a consortium. At least three species exhibited promising characteristics for serving as PGPY in enhancing zucchini growth. One important issue that emerged from this study is the necessity of investigating ecosystems beyond the rhizosphere or phyllosphere of plants as potential sources of PGPYs. Finally, the success of inoculation, depend not only on the quantity and quality of applied microbes and their adaptability, but also on the addition of organic substance as a key element for the intended action of inoculation. Given the recent recognition of yeasts' potential in promoting plant growth, further comprehensive studies are required to effectively evaluate and apply PGPYs as a means support for sustainable agriculture.

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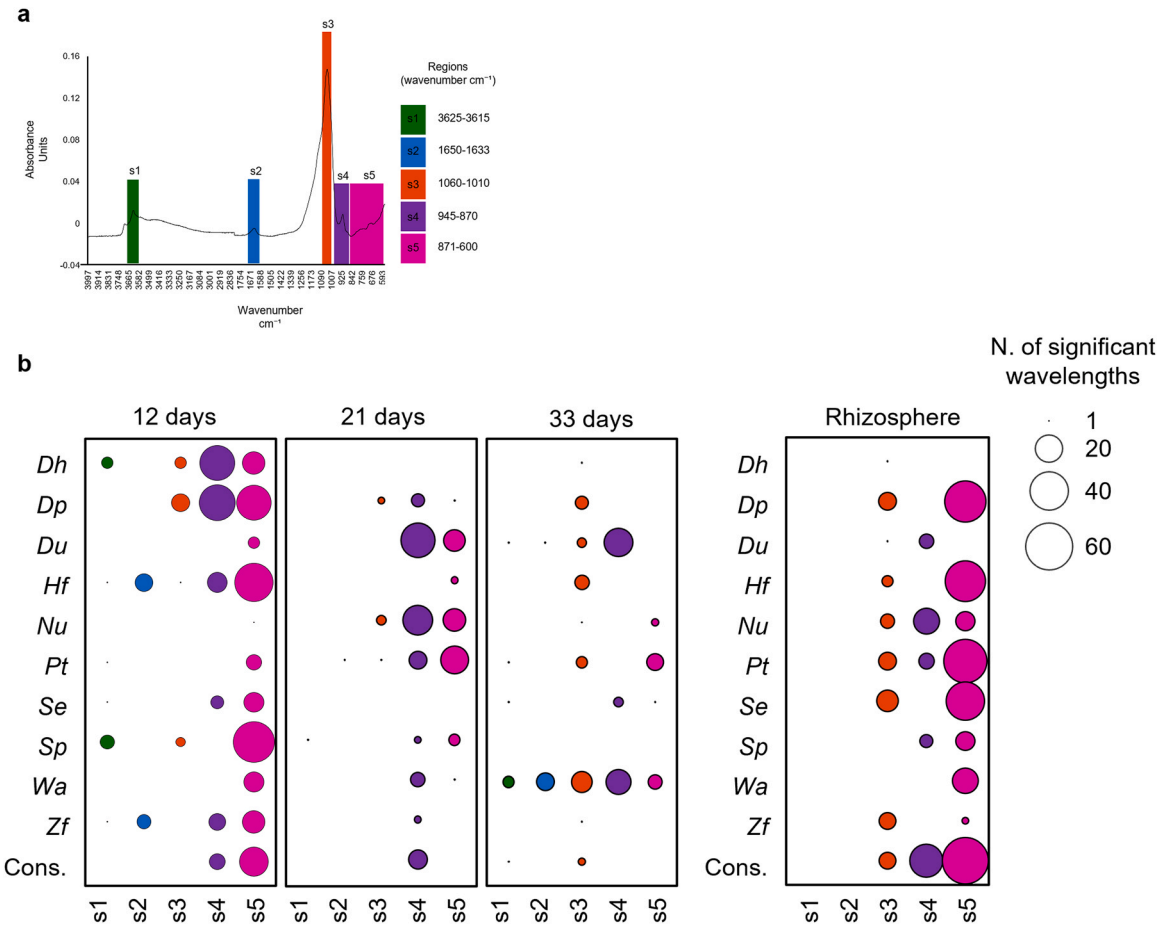

Fig. 3. Soil meta-metabolome analysis. The number of spectral wavelengths that significantly differed from the control ($p < 0.01$) (n=3), was separately calculated for each specific spectral area highlighted in panel (a), namely s1, s2, s3, s4 and s5. The effect yeast addition on the metabolomic profile of soil was analysed for samples of bulk (12, 21 and 33 days) and rhizosphere soil, as reported in panel (b). The size of bubbles, colored according to the respective spectral region, is proportional to the number of significant wavelengths detected.

Author statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.cpb.2024.100357.

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