Contrasting germination behavior of nodding broomrape towards soybean seeds- and sprouts-extract is associated with their corresponding phytohormones, sugars and isoflavones contents

Raman Manoharlal

ramanpdf01@gmail.com

ITC Life Sciences and Technology Centre https://orcid.org/0000-0001-7895-9893

G.V.S. Saiprasad

ITC Ltd

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Abstract

In the present study, a comparative study involving soybean (*Glycine max* L.) seeds- (SPE) and sprouts-extract (SSE) as a pre-conditioning media was performed to evaluate their allelopathic impact on nodding broomrape (*Orobanche cernua* L.) germination. Contrasting germination behaviour of broomrape, characterised by respective inhibition and induction in its germination was observed in response to an optimised concentration of SPE and SSE. Further study was conducted to explore the phyto-constituents of soybean seed- (SSP) and sprout-flour (SSF) responsible for differential germination of broomrape. Plant-growth regulators quantification revealed a relative enhanced bioactive gibberellin (GA_4) to abscisic acid (ABA) ratio (GA_4 : ABA) in SSF. Carbohydrates analysis revealed a relative reduced content of soluble-sugars, starch, sucrose (SUC) and raffinose-family oligosaccharides (RFOs) in concomitant with relative enhanced levels of reducing-sugars, glucose (GLU) and galactose (GAL) in SSF. The isoflavone content (IFC) analysis revealed a relative enhanced level of total IFC and individual bioactive aglycones [*viz.* daidzein, glycitein and genistein (GEN)] in SSF. Exogenous applications of GA_3, ABA and its antagonists (*viz.* uniconazole and fluridone), mono- (*viz.* GLU and GAL), oligo-saccharides (*viz.* SUC and RFOs), SPE_{50} and SSE_{50} pre-treated with enzyme-mix harbouring RFOs- and SUC-metabolizing enzymes (*viz.* α-galactosidase + invertase) and GEN as a pre-conditioning media of broomrape were also in agreement with the aforesaid observations. To the best of our knowledge, this is first report mentioning the contrasting germination behavior of broomrape towards SPE and SSE. Overall, these findings could be explored to formulate the ‘Green’ methods for the control of parasitic-weed infestation in agonomically important crops.

Introduction

The obligate root-, holo- and phanerogamic-parasitic weed, broomrape (genera *Orobanche / Phelipanche*; family *Orobanchaceae*), parasitises the crops and pastures from a diverse-range of plant-families *viz. Apiaceae*, *Asteraceae*, *Brassicaceae*, *Fabaceae* / *Leguminosae* and *Solanaceae* (Musselman and Parker 1982). The economically important and host-specific plants of broomrape comprise mainly of tobacco, mustard, tomato, potato, egg-plant, capsicum, onion, cabbage, cauliflower, turnip, canola, celery, hemp, lettuce, sunflower, linseed, spinach, chickpea, beans, lentils, flax, burr medic, clover and melon etc. to name a few (Das et al. 2020). Considering the devastating damages (0-100% crop-failures) and geographical-distribution [with predominance in warmer- and drier-regions of Asia, Central and Eastern Europe and Mediterranean (North Africa, the Middle East and Southern Europe)], a global economic-losses in the range of $1.3–2.6 billion per annum has been estimated due to the broomrape infestation (Das et al. 2020). Owing to achlorophyllous nature, predominant subterranean-life, small seed-size (0.3×0.2 mm), fecundity (> 5,00,000 seeds per plant), longevity (up to 15–20 years) and prevalence of huge seed-bank (up to 4 million seeds per m^2) in infested-fields, the existing control-strategies (e.g. cultural-practices, herbicide-applications and use of resistant host-varieties etc.) were not able to address the problem of broomrape infestation completely (Fernández-Aparicio et al. 2016, Das et al. 2020, Fernández-Aparicio et al. 2020).

The use of trap-crops have been recommended as an effective tool in mitigating the devastating damages caused by broomrape-infestation (Aksoy et al. 2016, Sarkar et al. 2018). Trap-crops functions primarily by inducing the broomrape germination without themselves being parasitized, thereby forcing the broomrape seedlings to die due to lack of nutritional-support, a process termed as 'suicidal-germination' (Zwanenburg et al. 2016b). The practical implication of suicidal-germination has been successfully demonstrated by inter-cropping of various main-crops with their corresponding trap-crops (Ye et al. 2017, Ye et al. 2020). During the last decade, soybean has also emerged as a successful trap-crop for root parasitic-weeds [e.g. sunflower broomrape, *O. cumana* Wallr. and *S. hermonthica* (Del.) Benth] (Zhang et al. 2012, Zhang et al. 2013). Research on soybean germination process gives an indication of existence of diverse-arrays of primary- and secondary-metabolites that could serves as potential allelochemical(s) in weed-control management (Mostafa et al. 1987, Manoharlal and Saiprasad 2019, Schandry and Becker 2020). Nevertheless, only circumstantial evidences provides a very limited insight of allelopathic-impact and associated allelochemical(s) of soybean on broomrape seed germination (Ma et al. 2012, Zhang et al. 2013). With this background information, a series of laboratory-experiments were performed to evaluate the allelopathic-potential of soybean seeds- (SPE) and sprouts-extract (SSE) on *in vitro* seed-germination of nodding broomrape (*O. cernua* L.). The aim of this study was to: (1) compare the allelopathic impact of SPE and SSE, (2) identify the responsible soybean phyto-constituent(s) and (3) validate the identified soybean phyto-constituent(s) as potential allelochemical(s) of broomrape germination.

Materials and methods

Chemicals and reagents

Synthetic analog of strigol, GR_{24} (Catalogue#racGR24) was purchased from Strigolab S.r.l., Turin, Italy. α-galactosidase (10,000 GAL units/g; #α-GAL) was purchased from Alferm Biotec, Bengaluru, India. GA_4 (#G7276), ABA (#A4906), yeast invertase (INV; ≥300 units/mg solid, #I4504), HPLC-grade sugar standards: D-(+)-glucose (#G8270), D-(+)-galactose (#G0750), D-(−)-fructose (#F0127), sucrose (#S7903), D-(+)-raffinose (#R0514), stachyose (#S4001), verbascose (#56217), daidzein (#D7802), glycitein (#G2785) and genistein (#G6649) and other analytical-grade reagents (AR) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Purity (≥ 90%) and functional-activity of these reagents were tested periodically by respective vendors on lot-to-lot basis.

Soybean seeds and sprouting conditions

Commercial soybean var. 'JS9560' was procured from ICAR-Indian Institute of Soybean Research (IISR), Indore, Madhya Pradesh, India. About 100 g of cleaned and surface-sterilized seeds with 0.3% (*v/v*) sodium hypochlorite (NaOCl) per batch were soaked in 500 ml of potable H_2O for 4 h with a constant shaking at 10 rpm in a customised motor seed dressing drum (GMW, Ambala, India), followed by draining and rinsing with distilled water (DW). Subsequently, imbibed seeds were divided equally in two-halves. One half is stored for the seed study. Other half of seed-lot is preceded for sprout formation,

wherein imbibed seeds were distributed evenly on a single layer of filter-paper in a sterile germination-tray. Each germination-tray was wrapped with a muslin-cloth (to allow entry of oxygen for the germinating seed while minimizing the contamination during the test-period) and placed in the customised seed-germinator (Acmas technologies Pvt. Ltd., India) at 30°C with 100% relative humidity (RH) for 3 days in the absence of light (Raman and Saiprasad 2018). Germination-trays were watered daily with DW during the course of germination. Physiological germination in terms of visible radicle protrusion (≥ 2 mm) was assessed over a test-period of 3 days, wherein no further radicle emergence was noted. Soybean sprouts harvested after 3rd day of germination were subjected to drying in a hot-air oven incubator (Inlab equipment; 230 volts, 5.4 Ampere) at 55°C to a moisture content of ≤ 5%. Dried-seeds as well as sprouts flours (hereafter referred as SPF and SSF, respectively) were milled using analytical grinder-mill, passed through a 0.6 mm sieve to obtain a fine-powder of 500 µm particle-size.

Soybean seeds- and sprouts-extract preparation

Approximately 250 mg of aforementioned SPF and SSF were extracted with 4 ml of 80% ethanol-water in a hot-water bath at 100°C with a magnetic-stirrer for 45 min. The slurry centrifuged for 30 min at 10,000 rpm and supernatant was collected. The extraction step was repeated with 2 ml of 80% ethanol-water and the recovered supernatants were pooled. The pooled-extracts were reduced to a volume of 2.5 ml using a rotary vacuum evaporator at 70°C to evaporate the ethanol. The corresponding concentrated supernatant from SPF and SSF (hereafter referred as SPE and SSE, respectively) was filtered through a 0.45 µm membrane (Millipore, Bedford, MA), diluted with sterile DW to the desired concentrations i.e. 10%, 25%, 50%, 75% and 100% and stored in glass-vials. Notably, SPE, SSE and theirs corresponding dilutions were prepared freshly before use to prevent the degradation of inherent bioactives (*if any*) prior to analysis.

Broomrape seed material

O. cernua seeds were collected from the mature flowering spikes of nodding broomrape plants parasitizing cultivated tobacco [*Nicotiana tabacum* L., Northern Light Soil (NLS) region, Rajahmundry, Andhra Pradesh (A.P.), India] during the growing season of 2021. The harvested seeds were stored in dark at 25°C until use. Before use, seeds were surface-sterilized with 0.3% (*v/v*) NaOCl for 5 min, rinsed extensively (three times for 5 min) with sterile DW and air-dried at room temperature.

Broomrape seed-germination assay

Broomrape seed-germination assay was performed as described previously (Louarn et al. 2012) with certain modifications. DW and filter-papers were sterilised by autoclaving before use. Heat-sterilised 10 mm glass filter-paper disc, GFFPD (Whatman GF/D; GE Healthcare Life Sciences) placed in a 50 mm petri-dish lined with two layers of 47 mm filter-paper (Whatman) was moistened with 1.5 ml of freshly prepared and specific concentration (i.e. 10%, 25%, 50%, 75% and 100%) of either SPE (hereafter referred as SPE_{10}, SPE_{25}, SPE_{50}, SPE_{75} and SPE_{100}) or SSE (hereafter referred as SSE_{10}, SSE_{25}, SSE_{50}, SSE_{75} and SSE_{100}) and subsequently dried in a laminar-flow for ~ 90 min. Approximately 50 surface-sterilized seeds were sown on each SPE or SSE pre-treated GFPPD in a new petri-dish lined with filter-paper and wetted

with 1.5 ml of DW. Four GFFPD with seeds were placed in each petri-dish. *O. cernua* seeds conditioned in the presence of specific concentration of SPE or SSE was referred as conditioned-treated seeds (CT). For comparative analysis, broomrape seeds were also pre-conditioned on an untreated GFFPD in a petri-dish lined with two layers of filter-paper and wetted with 1.5 ml of DW (hereafter referred as conditioned-untreated seeds, CU) or specific solvents of test-reagents (hereafter referred as solvent-controls, *described ahead*). The petri-dishes were sealed with parafilm, wrapped in aluminium-foil and incubated in dark at 25°C for 7 days. Following the conditioning-period, GFFPD with pre-conditioned CU and CT seeds were blotted to remove the excess water, spread on fresh GFFPD, transferred to a new petri-dish lined with two layers of filter-paper and wetted with 1.5 ml of synthetic germination stimulant (GS), GR_{24} (10 mg/L) or with 0.1% acetone (solvent-control). Unconditioned (U) seeds on GFFPD in a petri-dish lined with two layers of filter-paper and wetted with 1.5 ml of 10 mg/L GR_{24} were also included. The petri-dishes were sealed with parafilm, wrapped in aluminium-foil and further incubated in dark at 25°C for 3 days. To count the numbers of germinated seeds, U, CU and CT were examined and scored under 4× compound microscope (Leica DM1000 LED, Leica Microsystems, GmbH, Wetzlar, Germany). A bromrape seed had been considered as germinated when the visible radicle/germ-tube protruded from the testa. Germination was assessed as the cumulative germination-rate (CGR, in %) of seeds with an emerged radicle and base-corrected with the average seed-viability (%) obtained by 2,3,5-triphenyl tetrazolium chloride (TTC) analysis (Aalders and Pieters 1985).

Estimation of soybean GA_4 and ABA content

Endogenous levels of soybean plant-growth regulators (PGRs), GA_4 and ABA were quantified using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method as described earlier with slight modifications (Oulkar et al. 2011). *Sample preparation*. About 1 g of SPF and SSF was suspended in 5 ml of extraction-solvent [100% methanol (MeOH) harbouring 1% (*v/v*) formic acid (FA)]. MeOH-FA suspension was vortexed for 15 min, followed by centrifugation at 4,000 rpm for 10 min. The supernatant was collected and residue was re-extracted with 1 ml of extraction-solvent. The resulting supernatant from both the extractions were pooled and filtered through a pre-conditioned (with 1 ml of extraction-solvent) syringe filter (0.22 µm, PVDF, 33 mm). The resulting eluate was subjected to LC-MS/MS analysis. *LC-MS/MS instrumentation and conditions.* Chromatographic resolution was performed using an LC-MS/MS system (Shimadzu 8045) equipped with solvent pump, an auto-sampler, a gradient programmer, C-18 (octadecayl silane) column (150 mm × 4.6 mm × 5 µm, *Agilent Zorbax*) and a triple quadrupole tandem mass spectrometer (Electron multiplier detector, Shimadzu). The column temperature was maintained at 40°C. The injection volume of 20 µl was calibrated with needle wash for 6s. The resolution and elution of designated PGRs were accomplished by employing a binary-gradient mode (solvent A: 5 mM ammonium acetate with 0.05% FA in H_2O and solvent B: 100% acetonitrile (ACN) at a flow-rate of 0.5 ml/min for 18 min). The run program of solvent-system (% solvent A/B) was as follows: 0–5 min (95/5) and 12–18 min (20/80). A stock solution (1 mg/ml in MeOH) of HPLC-grade GA_4 and ABA were used as standard. Working solution of GA_4 and ABA standard at concentration of 100 µg/ml was subjected to linear calibration-curve analysis in a concentration range of 10–200 ng/ml. Data

was obtained by collecting the multi-reaction monitoring (MRM) mode. The area underneath the full-scan peak corresponding to more abundant accurate *m/z* of each molecule was used to calculate the relative response of each standard at each tested concentration. Retention-time (RT) of individual standard was used to identify the corresponding peaks from the LC-MS/MS chromatograms for each test-sample. The endogenous concentration of GA_4 and ABA was calculated after superimposing the sample chromatogram on their corresponding standard curve and expressed as ng/g on dry weight (d.w.) basis.

Estimation of soybean soluble-sugar (SS), reducing-sugar (RS) and starch (STA) content

Soybean SS, RS and STA content was measured calorimetrically based on spectrophotometric measurement using UV/Vis microplate and cuvette spectrophotometer (Thermo Scientific™ MultiskanTM GO). Approximately 50 mg of SPF and SSF was extracted using 1.5 ml of 80% (*v/v*) ethanolic solution as per the assay conditions described earlier (Jeong et al. 2010). The supernatant obtained was used for estimation of SS and RS, whereas the resulting pellet was used for extraction and estimation of STA.

SS extraction and estimation. Soluble carbohydrates were calorimetrically evaluated using anthrone reagent (Hansen and Møller 1975). An aliquot (200 µl) of aforesaid extract-solution was taken and final volume was made up to 1 ml with DW, followed by addition of 4 ml of anthrone reagent (200 mg in 95% H_2SO_4). Blank was performed using DW only. The test solution was vigorously shaken and kept in boiling water-bath for 8–10 min until a green to dark-green colour developed. After cooling the samples to room temperature, absorbance was recorded at 630 nm. The concentration of total SS in the test-samples were calculated from the calibration plot using standard curve of D-glucose (0-100 µg/ml) and expressed as percentage (%).

RS extraction and estimation. RS were estimated with 3,5-dinitrosalicylic acid (DNSA) reagent (Miller 1959). One ml of extract-solution was taken and final volume was made up to 3 ml with DW, followed by addition of 3 ml of DNSA reagent (200 mg in 95% H_2SO_4). The blank was performed using DW only. The test-solution was vigorously shaken and kept in boiling water-bath for 5 min. When the contents of tubes were still warm, 1 ml of 40% Rochelle salt solution was added. Samples were allowed to cool down and intensity of dark-red colour developed was measured at 510 nm. The concentration of RS in the test-samples were calculated from the calibration plot using standard curve of maltose (0-100 µg/ml) and expressed as %.

STA extraction and estimation. The resulting pellet from the aforesaid ethanolic-extract preparation was re-suspended in a digestion-mixture harbouring 0.65 ml of 52% perchloric acid ($HClO_4$) + 0.5 ml of DW and subjected to digestion at 0°C for 30 min. Following this, the content was centrifuged at 10,000 g for 10 min and supernatant was collected. The residual pellet was further extracted with same volume of digestion-mixture. Supernatants collected from both the extractions were pooled and neutralized with sodium carbonate. Final extraction volume was adjusted to 2.5 ml with DW. STA was estimated using

anthrone reagent as described above (Hansen and Møller 1975). STA concentration in each test-sample was calculated from the calibration plot using standard curve of D-glucose (0-100 µg/ml), multiplied by a factor of 0.9 and expressed as %.

Estimation of soybean mono- and oligo-saccharides content

Soybean mono-saccharides [*viz.* glucose (GLU), galactose (GAL) and fructose (FRU)] and oligo-saccharides [*viz.* sucrose (SUC) and RFOs] content were evaluated by High Performance Liquid Chromatography (HPLC) method as described earlier with certain modifications (Raja et al. 2015). *Sample preparation*. Approximately 75 mg of SPF and SSF was extracted twice with 20 ml of 80% ethanol-water in a hot water-bath at 55–60°C with a magnetic-stirrer for 45 min. Samples were centrifuged at 10,000 rpm for 30 min and supernatant was collected. The pooled-supernatant was reduced to a concentrated sugar-syrup by evaporating the ethanol in a rotary vacuum evaporator at 70°C, re-dissolved in 5 ml of DW and filtered through a 0.45 µm membrane (Millipore, Bedford, MA) into a 1.5 ml HPLC vial with a rubber slit septum and stored at 4°C till further use. *HPLC conditions and instrumentation*. An HPLC system (Agilent 1200) equipped with an auto-sampler, a gradient programmer, a solvent-pump and a refractive-index detector was used. The chromatographic column used was a sugar-pak I column (WAT085188, Waters Corporation, Waters India Pvt. Ltd.) with an internal dimension of 6.5×300 mm, filled with micro-particulate size (10 µm) of cation-exchange gel in calcium form. The mobile-phase consists of 50 mg/ml solution of $CaNa_2EDTA$. Operating conditions includes a flow-rate of 0.2 ml/min at an ambient temperature. 50 µl aliquots of aforesaid filtered-extract were injected into the mobile phase of HPLC via an auto-sampler to record chromatograms. Detection was done by measuring the change in refractive index of the column effluent passing through the flow-cell. All chromatograms were re-ordered on Agilent chemstation software. HPLC-grade sugar standards: GLU, GAL, FRU, SUC, verbascose (VER), stachyose (STA) and raffinose (RAF) were dissolved at 5 mg/ml in H_2O and subjected to HPLC analysis in a concentration range of 0-100 µg/ml. 50 µl aliquot of each standard was injected into the chromatographic system and the resulting peak-areas were plotted against concentration for the linear calibration curve. RT of each standard was used to identify the corresponding peaks on the HPLC chromatograms of test-samples. Peak-area was quantified by Chemstation software (Agilent). The relative concentration of individual sugar was calculated after superimposing the chromatogram of sample on their corresponding standard curve. The concentration of GLU, GAL, FRU, SUC, total and individual RFO component were expressed as g/100 g on d.w. basis. Notably, individual RFO sugars (*viz.* VER, STA and RAF) concentration was summed to compute the total RFOs levels.

Estimation of soybean isoflavones content (IFC)

An HPLC based quantitative estimation of soybean IFC, wherein acid-hydrolysis of 12 endogenous IF isomers to their respective aglycone IF (AIF); daidzein (DAI), glycitein (GLY) and genistein (GEN) was evaluated as described earlier with certain modifications (Raja et al. 2015). *Sample preparation.* Approximately 250 mg of SPF and SSF was extracted twice with 5 ml of 80% ethanol, followed by acid-

hydrolysis with 1 ml of concentrated HCl in a boiling water-bath at 80°C for 2 h. The supernatant obtained after centrifugation at 10,000 rpm for 10 min was collected and passed through a syringe-filter (0.45 µm, PVDF, 33 mm). The resulting eluate was stored at 4°C until subjected to HPLC analysis. *HPLC instrumentation and conditions.* A 20 µl aliquot of aforesaid prepared sample was injected into HPLC system (Agilent 1200, equipped with an auto-sampler, a gradient programmer, a solvent pump and a diode array detector) housing a C-18 silica column (5 µm with dimension of 250 × 4.6 mm; Inertsil ODS 3V). The column temperature was maintained at 40°C. The elution and resolution of AIFs was achieved by a binary-gradient solvent system [solvent A: 10% ACN and solvent B: 38% ACN] at a flow-rate of 0.8 ml/min for 25 min. The run program of solvent system (solvent %: A/B) was as follows: 0 min (0/100), 5 min (10/90), 20 min (0/100) and 25 min (0/100). Stock solution (1000 µg/ml) of HPLC-grade AIF standards; DAI (in dimethyl sulfoxide, DMSO), GLY (in dimethylformamide, DMF) and GEN (in DMSO) were dissolved in corresponding solvent and were subjected to HPLC in a concentration range of 0–10 µg/ml. The resulting peak-area was plotted against standard concentration for the linear-calibration curve. The RT of individual standard was used to identify the corresponding peak from the HPLC chromatograms for both SPF and SSF. DAI resolution was detected at 250 nm, while that of GLY and GEN was detected at 260 nm. The relative concentration of individual IFC was calculated after superimposing the sample chromatogram on their corresponding standard-curve and expressed as mg/kg on d.w. basis. The concentration of DAI, GLY and GEN were summed to compute the total IFC (mg/kg).

Effects of specific reagents on in vitro seed-germination of broomrape

To study and validate the impact of specific phyto-constitutent as a potential allelochemical, broomrape seed-germination assay was performed as described above except that instead of DW, the test-reagents i.e. GA_3 (50 mg/L), ABA (300 µM), uniconazole (UNI; 0.5 mg/L), fluridone (FL; 50 mg/L), GLU (10 mM), GAL (10 mM), FRU (10 mM), SUC (10 mM), RAF (10 mM), STAC (10 mM), VER (10 mM), DAI (50 µM), GLY (50 µM) and GEN (50 µM) were tested as a pre-conditioning media for 7 d. To study the impact of pre-treatment with RFOs- and SUC-metabolizing enzyme, 10 g of fine-ground SPF and SSF were incubated with 100 ml (flour: assay-buffer = 1:10) of 0.1 M sodium acetate buffer (pH 5.0) at room temperature for 20 min to obtain a uniform slurry. The resulting slurry was supplemented with an enzyme-mix harbouring 100 U/ml α-galactosidase (α-GAL, E.C. 3.2.1.22) + 15 U/ml invertase (INV, E.C. 3.2.1.26) and further incubated at 50°C for 3 h with continuous shaking at 50 rpm in a rotary-shaker. The corresponding untreated-control (without addition of α-GAL + INV mix) sample was treated with equivalent volume of DW only. After incubation at an indicated time-point, samples were filtered through Whatman No. 1 filter-paper, dried under vacuum at 40°C for 2 h, evaluated for mono- and oligo-saccharides content in concomitant with aforementioned ethanolic-extract preparation, followed by their testing as pre-conditioning media on broomrape seed-germination as described above. In all cases, broomrape germination test was performed in triplicates and repeated at least three times.

Data analysis

Results were expressed as means ± standard deviations (SDs). Analysis of variance (ANOVA) was used to analyse the significant ($p \leq 0.05$) differences between groups.

Results

Soybean seeds- and sprouts-extract preparation

Soybean seeds were subjected to lab-scale sprouting under optimized germination-conditions (30°C, 100% RH and dark) wherein a cumulative germination-rate (CGR) of ~ 76% was observed at the end of test-period (3rd d) (Fig. 1*inset*). A fine and free-flowing soybean sprouts-flour (SSF) with moisture-content of ≤ 5%, bulk- and tapped-density of 0.37 and 0.66 g/ml respectively, was prepared from the harvested sprouts (Fig. 1). Likewise, a high-quality soybean seeds-flour (SPF) with a characteristic moisture-content of ≤ 5% and a respective bulk- and tapped-density of 0.38 and 0.69 g/ml was also prepared from the soybean seeds (Fig. 1). Subsequently, ethanolic-extract of soybean seeds- and -sprouts (*viz.* SPE and SSE) was prepared from the aforesaid SPF and SSF, respectively. A series of concentration (i.e. 10%, 25%, 50%, 75% and 100%) were prepared from SPE (hereafter referred as SPE_{10}, SPE_{25}, SPE_{50}, SPE_{75} and SPE_{100}) and SSE (hereafter referred as SSE_{10}, SSE_{25}, SSE_{50}, SSE_{75} and SSE_{100}), followed by their testing on *in vitro* seed-germination of broomrape. Each extract was prepared freshly before use to prevent the degradation of inherent bioactive prior to analysis

SPE_{50} and SSE_{50} as a pre-conditioning media demonstrates an optimum and contrasting impact on broomrape seed-germination

At foremost, broomrape seeds-viability was interpreted by TTC test, wherein an average seed-viability of ~ 75% was observed (Fig. S1). The observed seed-viability (%) was used as a normalization-factor in calculating the broomrape CGR throughout the present study. In all cases, CGR was assessed by counting the number of seeds with an emerged radicle at the end of test-period (7 days) under prescribed assay-conditions. As expected, we observed that unconditioned (U) seeds did not show any germination response following stimulation with GR_{24} (Fig. 2). On the other hand, the CGR of broomrape seeds pre-conditioned in DW (hereafter referred as CU) followed by stimulation with GR_{24}, was observed to be ~ 76% (Fig. 2). The specificity of GR_{24} was validated by using its solvent [0.1% acetone (*v/v*)] as GS, wherein no germination was observed for *O. cernua* (Fig. S2, *SC bar*). With this background work, firstly we ought to determine the direct-impact of SPE and SSE as a GS on *O. cernua* seed-germination. Interestingly, no direct-effect of SPE as well as SSE was observed on *O. cernua* seed-germination at all tested concentrations (Fig. S2). On the contrary, an intriguing germination behaviour of broomrape seeds was observed upon its pre-conditioning in the presence of SPE and SSE (hereafter collectively referred as CT), followed by stimulation with GR_{24}. For SPE, a CGR of 72%, 67%, 55%, 55% and 55% was observed in the presence of SPE_{10}, SPE_{25}, SPE_{50}, SPE_{75} and $SPE_{100,}$ respectively (Fig. 2). Comparative analysis (CU *vs* CT) revealed a significant and progressive decline in *O. cernua* CGR to the extent of 5%, 12% and 27% at SPE_{10}, SPE_{25} and SPE_{50} respectively, followed by reaching a plateau (27% decline) at SPE_{75} and

SPE_{100} (Fig. 2). Regarding SSE, a CGR of 78%, 83%, 93%, 70% and 58% was observed in the presence of SSE_{10}, SSE_{25}, SSE_{50}, SSE_{75} and $SSE_{100,}$ respectively (Fig. 2). As evident, comparative analysis (CU *vs* CT) revealed a significant and progressive increment of 2%, 9% and 22% at SSE_{10}, SSE_{25} and SSE_{50} respectively (Fig. 2). However, at SSE_{75} and SSE_{100}, *O. cernua* CGR was reduced to the extent of ~ 8 and 24%, respectively (Fig. 2). In order to evaluate whether the observed inhibitory effect at all tested-concentration of SPE and higher concentrations of SSE (i.e. SSE_{75} and SSE_{100}) involved certain degree of toxicity towards *O. cernua* seeds, we measured the viability of corresponding CT seeds. Indeed, no effect on seed-viability at all tested concentration of SPE and SSE was detected (Fig. S1). These results also indicate that SPE as well as SSE at all tested concentrations does not alter the intrinsic ability of *O. cernua* seeds to respond to GR_{24}. Notably, an optimum suppressive- and stimulating-effect on *O. cernua* CGR was observed at SPE_{50} and SSE_{50}, respectively (Fig. 2, *marked bars*), henceforth our further study was focused to evaluate the soybean phyto-constituent(s) that could be responsible for the distinguished and contrasting behaviour of *O. cernua* seeds at this specified concentration of SPE and SSE.

SPF and SSF possesses a relatively low and high GA_4 : ABA ratio, respectively

The content and ratio of GAs and ABA are the key determinants for soybean seed-germination (Shuai et al. 2017). Taking a cue from this, firstly we ought to determine the endogenous levels of bioactive GA (i.e. GA_4) and ABA in SPF and SSF. We observed a relative enhanced level of GA_4 (RFC = 1.65) in SSF in contrast to SPF (Fig. 3a). On the contrary, a reduced level of ABA (RFC = -1.46, negative sign indicates the reduction) was observed in SSF in contrast to SPF (Fig. 3b). Consequently, an obvious higher GA_4 : ABA ratio (RFC = 2.41) was observed in SSF than SPF (Fig. 3c). These results also corroborate the existing notion that soybean seed-germination (or any seed-germination process *per se*) is regulated by antagonism between GAs and ABA biosynthesis- and signalling-pathways (Tuan et al. 2018). These observations reflect that SPE and SSE exert their action on nodding broomrape seed-germination possibly through their corresponding enhanced endogenous levels of GA and ABA. However, the probability of other PGRs as well as other factors (*described ahead*) that influences the broomrape seed-germination can't be ignored.

SPF and SSF possesses a contrasting level of STA, SS, RS, GLU, GAL, SUC and RFOs

Seed-germination, being a complicated process, involves a series of primary metabolic- changes, wherein carbohydrates serves as a major-energy source (Ali and Elozeiri 2017). To elucidate the carbohydrates dynamics in SPF and SSF, the physiological levels of starch (STA), soluble-sugars (SS), reducing-sugars (RS), mono-saccharides [*viz.* glucose (GLU) and galactose (GAL) and fructose (FRU)] and oligo-saccharides [*viz.* sucrose (SUC) and raffinose-family oligosaccharides (RFOs)] were evaluated in our further investigation. Our calorimetric estimation revealed a significant decline in level of STA (RFC = -1.77; 1.03 *vs* 0.58 g/100g) and SS (RFC = -1.46; 7.63 *vs* 5.23 g/100g) in SSF in contrast to SPF (Fig. 4a

and *b*). On the contrary, comparative analysis reveals a relative enhanced level (RFC = 2.41; 0.34 *vs* 0.81 g/100g) of RS in SSF as compared to SPF (Fig. 4c). An HPLC based analysis was performed to evaluate the levels of major mono- (*viz.* GLU, GAL and FRU) and oligo-saccharides (*viz.* SUC and RFOs i.e. VER, STA and RAF) in SPF and SSF. A representative HPLC chromatogram depicts the resolution, RT (in min) and corresponding linear equation obtained from the calibration-curve of each sugar standard i.e. VER, STA, RAF, SUC, GLU, GAL and FRU (Fig. S3*a*). A linear-correlation between its concentration (0-100 µg/ml) and peak-area along with a typical determination-coefficient of > 99% was obtained for each sugar-standard (Fig. S3*b*). From the complex-mixture of ethanolic sugar-syrup of SPF and SSF, our HPLC procedure was able to resolve and clearly distinguish all the aforesaid 7 sugars within RT of 10–30 min (Fig. S4). Comparative quantitative analysis of mono-saccharides revealed a significant increment in GLU (RFC = 2.26; 0.27 *vs* 0.61 g/100g) and GAL content (0.06 g/100 g *vs n.d.*) of SSF in comparison to its SPF counterpart (Fig. 4d). Notably, FRU level was not-detected (*n.d.*) in either of the test-sample (Fig. 4d). Comparative quantitative analysis of oligo-saccharides demonstrated a significant decline in SUC content (RFC = -41.21; 1.42 *vs* 0.03 g/100g) of SSF relative to SPF (Fig. 4d). Likewise, measurement of total RFOs revealed a significant decline (RFC = -4.94; 5.31 *vs* 1.09 g/100g) in SSF relative to SPF (Fig. 4e). Further evaluation of individual RFO component revealed a relative reduced level in SSF in contrast to SPF, wherein a respective RFC of -2.8 (0.11 *vs* 0.04 g/100g), -4.7 (4.2 *vs* 0.89 g/100g) and −6.27 (1.0 *vs* 0.16 g/100g) was observed for VER, STA and RAF, respectively (Fig. 4e).

SPF and SSF possesses a relatively low and high IFC, respectively

Our previous study demonstrated a relative enhanced antioxidant activity during soybean germination (Manoharlal and Saiprasad 2019). Isoflavones (IFs), being a primary physiologically bioactives in soybean, serves as first-line of antioxidant-defence to scavenge the reactive oxygen species (ROS) (Yoon and Park 2014, Ma et al. 2019). Notably soybean IFs primarily exist in its 3 bioactive aglycone IFs (AIFs) forms *viz.* DAI (4',7-dihydroxyisoflavone), GLY (6-methoxydaidzein) and GEN (4',5,7-trihydroxyisoflavone) (Lee et al. 2005). Henceforth, we were prompted to determine the endogenous levels of these 3 AIFs in SPF and SSF. A representative HPLC chromatogram depicts the resolution, RT (in min) and corresponding linear equation obtained from the calibration-curve of each of AIF standard i.e. DAI, GLY and GEN (Fig. S5*a*). For each AIF standard, a linear-correlation between its concentration (0–10 µg/ml) and peak-area with a typical determination-coefficient of > 99% was obtained (Fig. S5*b*). From the ethanolic- and acid-hydrolysed extract of SPF and SSF, we were able to clearly distinguish all the 3 AIFs within RT of 7–15 min (Fig. S6). Our results revealed a significant increment (RFC = 1.36) in total IFCs for SSF relative to SPF (Fig. 5, 223 *vs* 304 mg/kg). Further comparative evaluation of SSF relative to SPF revealed a respective increment in individual AIF component with a characteristic RFC of 1.38 (43 *vs* 60 mg/kg), 1.28 (128 *vs* 163 mg/kg) and 1.54 (52 *vs* 80 mg/kg) for DAI, GLY and GEN, respectively (Fig. 5).

Purified-reagent(s) as a pre-conditioning media partially mimics the identified soybean phyto-constituents in mediating the germination response of broomrape seeds

In a bid to substantiate the aforementioned claims made by identified soybean phyto-constituents in mediating the differential germination response of broomrape seeds, we were prompted to evaluate the impact of corresponding specific-reagents as a pre-conditioning media on broomrape germination-behaviour. A difference in broomrape CGR in the absence and presence (*if any*) of purified-reagent as a pre-conditioning media (hereafter collectively referred as CR) was measured as a functional-readout to determine their specific impact of broomrape seed-germination. For the same, firstly HPLC-grade PGRs i.e. GA_3 (25 mg/L) and ABA (300 µM) as pre-conditioning media were used that demonstrated a broomrape CGR of 96% and 13%, respectively (Fig. 6a). This observation was further complimented by use of corresponding antagonist of GA_3 and ABA i.e. uniconazole (UNI; 0.5 mg/L) and fluridone (FL; 50 mg/L) as a pre-conditioning media, wherein a respective broomrape CGR of 11% and 88% was observed (Fig. 6a). Comparative analysis (CU *vs* CR) revealed a significant change in broomrape CGR for + GA_3 (RFC = 1.27), +ABA (RFC = -5.7), +UNI (RFC = -7) and + FL (RFC = 1.16) with respect to CU (Fig. 6a).

Likewise, HPLC-grade sugars i.e. GLU (10 mM), GAL (10 mM), FRU (10 mM), SUC (10 mM), VER (10 mM), STAC (10 mM) and RAF (10 mM) were tested as broomrape pre-conditioning media, wherein a respective CGR of 88%, 83%, 72%, 80%, 46%, 48% and 51% was observed (Fig. 6b). Comparative analysis (CU *vs* CR) revealed that relative to CU, a noticeable change in broomrape CGR for + GLU (RFC = 1.15), +VER (RFC = -1.7), +STAC (RFC = -1.6) and + RAF (RFC = -1.5) (Fig. 6b). On the contrary, very marginal to not detectable (*n.d.*) changes in broomrape CGR was observed for + GAL (RFC = 1.09), +FRU (RFC = -1.05) and + SUC (RFC = 1.05) (Fig. 6b). The sugar-specific germination-signal perceived by broomrape was further corroborated by use of SPE_{50} and SSE_{50} as a pre-conditioning media that were prepared from the enzyme-mix [harbouring α-GAL (100 U/ml) + INV (15 U/ml] pre-treated SPF and SSF, respectively. A representative HPLC depicts the chromatographic resolution of untreated and enzyme-mix (harbouring α-GAL + INV) pre-treated sugar-extracts of SPF and SSF (Fig. S7). A significant increment in GLU (RFC = 2.22; 0.27 *vs* 0.61 g/100 g), with no detectable effect on GAL and FRU and a significant decline in its endogenous levels of SUC (RFC = -26; 1.42 *vs* 0.06 g/100 g), VER (RFC = *n.d.*; 0.11 g/100 g *vs n.d.*), STAC (RFC = -104; 4.2 *vs* 0.04 g/100 g), RAF (RFC = -18; 1 *vs* 0.05 g/100 g) and total RFOs (RFC = -50; 5.31 *vs* 0.09 g/100 g) was observed for enzyme-mix pre-treated SPF (Fig. 6c, *insets*). On the contrary, comparative analysis of enzyme-mix pre-treated SSF with its corresponding untreated counterpart revealed not significant differences in any of the sugar tested (Fig. 6c, *insets*). An observation of broomrape CGR in the presence of SPE_{50} and SSE_{50} derived from the aforesaid enzyme-mix pre-treated SPF and SSF as a pre-conditioning media revealed a respective CGR of 77% and 95%, respectively (Fig. 6c). In line with sugar analysis results, comparative analysis of enzyme-mix pre-treated SPE_{50} and SSE_{50} with their corresponding untreated counterpart revealed an increment (RFC = 1.23) in broomrape CGR for SPF only, without showing any significant impact by SSF (RFC = 1.02, Fig. 6c). Taken together, these results further substantiates the observations that GLU, SUC and RFOs played a critical and important role in broomrape seed-germination process.

For evaluating the specific impact of IFC, broomrape seeds were pre-conditioned in the presence of each of AIFs i.e. DAI (50 µM), GLY (50 µM) and GEN (50 µM), followed by stimulation with GR_{24} (10 mg/L)

under prescribed assay-conditions. A CGR of 73%, 74% and 87.5% were observed for DAI, GLY and GEN, respectively (Fig. 6d). Comparative analysis (CU *vs* CR) revealed no significant change in broomrape CGR in + DAI and + GLY (Fig. 6d). On the contrary, a significant increment (RFC = 1.15) in broomrape CGR was observed for + GEN (Fig. 6d). Representative micrographs depict the *O. cernua* seeds / germlings at the end of experiment (Fig. S8). Of note, the functional-specificity of each of aforementioned test-reagents were further corroborated by the fact that no difference in broomrape CGR was recorded for broomrape seeds pre-conditioned in the presence of either of their corresponding solvents [*viz.* 0.1% EtOH, 0.1% MeOH, 0.1% DMSO and 0.1% DMF (*v/v*)], under prescribed assay-conditions (Fig. S9).

Discussion

'Plant-allelopathy' is a natural interference mechanism between donor- and receptor-plants, wherein certain and specific allelochemicals (*a.k.a.* bio-communicators) released from the donor-plants exerts either positive- (e.g. shade-effect, weed-crop competition, crop-protection and re-establishment etc.) or negative-impact (e.g. soil-sickness, auto-toxicity and biological-invasion etc.) to conspecific and hetero-specific receiver's plants (Rice 2012). There are periodic evidences of *leguminosae* family plants *viz.* soybean (*G. max*), pea (*Pisum sativum*), winter vetch (*Vicia villosa*), velvet bean (*Mucuna pruriens*), alfalfa (*Medicago sativa*), chickpea (*Cicer arietinum*), red clover (*Trifolium pretense*) and cowpea (*Vigna unguiculata*) with potential allelopathic properties (Huber and Abney 1986, Qasem 1998, Yasmin et al. 1999, Akemo et al. 2000, Fujii 2001, Kato-Noguchi 2003, Xuan et al. 2003, Kamo et al. 2006, Xiao et al. 2006, Yan and Yang 2008, Narwal and Haouala 2013). Talking specifically about soybean, its allelopathic impact has been demonstrated in agronomical-field by its inter-cropping with cotton (*Gossypium hirsutum* L.) and maize (*Zea mays* L.), wherein a significant inhibition of their corresponding weeds i.e. purple nutsedge (*Cyperus rotundus* L.) and purple-/giant-witchweed (*Striga hermonthica* L.) infestation, in concomitant with enhanced yield of main-crops has been demonstrated (Iqbal et al. 2007, Odhiambo et al. 2011). In a few separate studies, soybean as a broad-spectrum allelopathic-plant has been authenticated by fact that its root-exudates promoted the growth of certain fungus (*Fusarium semitectum*, *F. oxysporum* and *Gliocladium roseum*), whereas its leaf-extracts inhibited the growth and germination-rate of Spear grass (*Imperata cylindrical* L.) (Ju et al. 2002, Chikoye and AK 2012). Nevertheless, aside from these circumstantial evidences, there is a very limited insight on allelochemicals responsible for soybean allelopathy against *Orobanche* sp. Henceforth, we explored soybean as a model-system to study its allelopathic impact and to identify the allelochemical(s) that could influences the seed-germination of nodding broomrape, *O. cernua*. For the same, a preliminary investigation involving the ethanolic soybean seeds- (SPE) as well as sprouts-extract (SSE) was performed to evaluate their impact (*if any*) on *in vitro* germination of nodding broomrape under laboratory-conditions (Fig. 1).

Broomrape germination, being reckoned as a two-step process, wherein a pre-requisite independent-phase *a.k.a.* 'conditioning-period' (to acquire the sensitivity to natural or synthetic germination-stimulant, GS) is followed by a host-specific parasitic-phase *a.k.a.* 'chemical-stimulation' (to initiate the germination process that ends with the radicle-protrusion) (Joel et al. 1991). Thereby, firstly we investigated the

impact of SPE and SSE as a pre-conditioning media (indirectly measured by recording CGR) as well as GS (directly measured by recording CGR) on *in vitro* seed-germination of *O. cernua*. We noticed that SPE and SSE, when applied as a GS, do not affect *O. cernua* seed-germination at all the tested- concentration (Fig. S2). This is indeed in contrast to earlier observations, wherein aqueous and/ methanolic-extract of soybean roots, shoots and leaves has been shown to induce the broomrape seed-germination (Ma et al. 2012, Zhang et al. 2012, Zhang et al. 2013). We speculated that this contradictory difference could be either due to tissue-specificity (whole-seeds and/ sprouts *vs* roots, shoots and leaves tissue) or due to differences in sample-processing, extraction procedure and/ solvents (ethanol *vs* aqueous / methanol) used or a combination of all. Moving forward and to our pleasant surprise indeed, a distinctly specific- and opposite-effects was observed when SPE and SSE were used as pre-conditioning media. For SPE, a significant and progressive decline in *O. cernua* CGR was observed at SPE_{10}, SPE_{25} and $SPE_{50,}$ followed by reaching a plateau at SPE_{75} and SPE_{100} (Fig. 2). On the contrary, a significant and progressive increment in *O. cernua* CGR was observed at SSE_{10}, SSE_{25} and SSE_{50}, which was followed by a slight decline at SSE_{75} and SSE_{100} (Fig. 2). Three inferences can be made from all these observations. Firstly, broomrape seed-germination response is growth-stage specific, wherein broomrape seeds responds quite differently to a particular exogenous-factor(s) when used either as pre-conditioning media or as GS. This observation could also be supported and linked with a previous study, wherein *O. cernua* seeds upon exposure to aqueous-extract of asthma-plant (*Euphorbia hirta*) as a pre-conditioning media and as a GS exhibited a respective inhibition and induction in its germination (Abdelhalim et al. 2018). Secondly, a distinguish specific- and contrasting-effect on broomrape seed-germination was observed upon its exposure to SPE and SSE as a pre-conditioning media. One hypothesis that can be anticipated to explain the differential germination behaviour of *O. cernua* is that SPE and SSE might possess either specific or certain common germination-affecting factor(s) in a different concentration that affects the sensitivity of *O. cernua* seeds towards GS in a contrasting-manner. Thirdly, both promotive- as well as inhibitory-effect on *O. cernua* CGR was observed at a specific-concentration of SSE (Fig. 2). Indeed, a similar kind of observation has been also made in a previous study, wherein undiluted and diluted root-exudates of soybean has been demonstrated to promote and inhibit the germination of cucumber (*Cucumis sativus* L.), respectively (Wang et al. 2009). The observed dual-effect of SSE on *O. cernua* CGR could be attributed to either dose-dependent effect of its prevalent bioactive(s) or existence of inhibitory-compound(s) that comes to the fore only at a higher concentration of SSE, which either act as GS antagonist or causes the specific inhibition in broomrape seed-germination process. Further detailed experiments are needed to validate these hypothesis and/ assumptions.

Notably, soybean (or any seed-germination process *per se*) involves a series of dynamic biochemical-, nutritional- and sensorial-changes in the seeds, characterised by fine-tuned homeostatic reprogramming of primary- (*viz.* carbohydrate, protein, fat and energy) and secondary-metabolites (*viz.* alkaloids, terpenoids, phytosterols, saponins, phenolics, fatty acid-derived substances, polyketides, non-ribosomal polypeptides and enzyme co-factors etc.) (Mostafa et al. 1987, Bau et al. 2000, Lee et al. 2013, Ma et al. 2018). During the germination process, soybean also secretes certain specific primary- (*viz.* sugars, organic acids, mono-, oligo- and poly-saccharides) and secondary-metabolites (*viz.* IFs) into the

rhizosphere either directly (as a root-exudates) or indirectly (through leaching, volatilization and microbial-decomposition of residues etc.) (Timotiwu and Sakurai 2002, Tawaraya et al. 2014, Tsuno et al. 2018, Sugiyama 2019). Till date, natural allelochemicals identified belongs to either non-nutritional primary- (*e.g.* fatty-acids and non-protein amino acids etc.) or secondary-metabolites (*e.g.* benzoquinones, flavonoids, terpenoids, triketones, coumarins, strigolactones (SLs), phenolic acids, tannins and lignin etc. to name a few) (He et al. 2019). Taking a cue from all these studies, we were promoted to connect these dots by exploring the physiological levels of potential alleleochemical(s) in SPF and SSF, with a major emphasis on soybean endogenous PGRs (GA and ABA), sugars (SS, RS, mono-, oligo- and poly-saccharides) and IFs.

Among an array of external- and internal-factors, one of the most important determinants that decide the fate (either germination or dormancy) of any seed is PGRs (Shu et al. 2016). PGRs includes GAs, ABA, ethylene, auxin, cytokinins, salicylic acid, brassinosteroids, jasmonates, nitric oxide, SLs and peptide-hormones (Santner et al. 2009). Among these, SLs as a natural GS of *Orobanche* and *Striga* has been studied extensively (Zwanenburg and Pospíšil 2013, Zwanenburg et al. 2016a, Banerjee and Bhadra 2020). Notably, orobanchyl acetate (a kind of SLs) has been also identified from soybean root-exudates (Xie et al. 2008). However, the probability of SLs as a bioactive component of SPE and/ SSE can be ruled out by the fact that unlike SLs, SPE and/ SSE as a GS did not directly induce the germination of conditioned broomrape seeds (Fig. S2). Herein, we speculate the probable involvement of PGR(s), other than that of SLs, for their differential effects of SPE and/ SSE on broomrape germination. Notably, a universal long-standing notion posits that GAs and ABA acts antagonistically and a paradigm shift in GAs : ABA homeostatic balance dictates the seed-germination process (Shu et al. 2016). It has been hypothesized that during conditioning-period, a series of proceedings involving bioactive GA biosynthesis, followed by ABA catabolism takes place that enables the broomrape seeds to become perceptive and responsive to subsequent host-specific SLs signalling events (Delavault 2015). Considering these facts, GA and ABA stakes their claims as a potential allelochemical(s) for differential mode of action in SPF and SSF. True to this notion and anticipation, our results indeed revealed a relative enhanced GA_4 : ABA ratio in SSF, wherein physiological level of GA_4 (the main bioactive GA in soybean) and ABA levels was relatively-higher and -lower respectively. To validate the GAs and ABA as functional allelochemicals in SSF and SSF, we further tested the impact of purified-grades of GA_3 and ABA on *O. cernua* germination. We observed that GA_3 (25 mg/L) and ABA (300 µM) as a pre-conditioning media of *O. cernua* were able to partially-mimics the respective germination-impact of SSE and SPE (Fig. 6a). Indeed, the functional involvement of GAs and ABA during broomrape conditioning has been already demonstrated in few independent studies, wherein exogenous application of GA_3 and ABA has been reported to stimulate and inhibit the germination of different *Orobanche* sp., respectively (Zehhar et al. 2002, Song et al. 2005, Song et al. 2006). Conversely, it has been also shown that biosynthesis inhibitors of GAs and ABA causes a significant decline and enhancement in the germination rate of broomrape, respectively (Chae et al. 2004). Herein, we used uniconazole (UNI, 0.5 mg/L) and fluridone (FL, 50 mg/L) as specific inhibitor of GA and ABA biosynthesis, respectively. UNI [(E)-1-(4-Chlorophenyl)-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl)pent-1-en-3-ol], a triazole plant growth retardant, has been reported to reduce the

endogenous levels of bioactive GAs by inhibiting the conversion of ent-kaurene to ent-kaurenoic acid catalysed by monooxygenases (Izumi et al. 1985). Moreover, UNI also showed side-effects in terms of enhanced physiological level of ABA mainly by inhibiting its oxidative catabolism catalysed by cytochrome P450-dependent monooxygenase (Saito et al. 2006). Likewise, FL [1-methyl-3-phenyl-5-(3-trifluoromethylphenyl)-4-(1H)-pyridinone], a commonly used aquatic-herbicide is generally used to systemic control the unwanted nuisance weeds (*viz.* duckweed, hydrilla and Eurasian watermilfoil etc.) in fresh-water bodies, mainly functions by blocking the carotenoid biosynthesis pathway *via* inhibiting the desaturation step of phytoene to phytofluene catalysed by phytoene desaturase (Chae et al. 2004). Carotenoids, being the main precursor of ABA, thereby FL application leads to reduced biosynthesis of downstream lying ABA in plants (Popova and Riddle 1996, Yoshioka et al. 1998). Seed-priming with FL has been also known to break seed dormancy in several plant species including parasitic (e.g. *S. asiatica* and *O. minor*) as well as non-parasitic [e.g. *Arabidopsis thaliana*, tobacco (*N. plumbaginifolia*), mung bean (*V. radiata*), Taiwan cherry (*Prunus campanulate*), ornamental peach (*P. persica*) etc. to name a few] seed germination (Thind 1997, Grappin et al. 2000, Ali-Rachedi et al. 2004, Kusumoto et al. 2006, Chen et al. 2007). Our results further substantiate and consolidate these facts, wherein UNI and FL as a pre-conditioning media of *O. cernua* showed an impact inverse to that of exogenous GA_3 and ABA application, characterised by a respective inhibition and induction in CGR of *O. cernua* (Fig. 6a). These results, thus corroborates and further establishes a prominent role of GAs and ABA in differential allelopathic impact displayed by SPE and SSE.

Like PGRs, sugars also serve as an important signature molecule that governs the seed germination and development (Eveland and Jackson 2012). Recent studies have demonstrated the role of inherent sugar-profile in broomrape seed-germination (Okazawa et al. 2020, Okazawa et al. 2022). On an average, soybean seeds comprise ~ 33–35% carbohydrates on d.w. basis (Hymowitz and Collins 1974), wherein sugar constitutes ~ 40–45% of the total carbohydrates that accounts for ~ 14% of dry matter (d.m.) (Grieshop et al. 2003). STA is a major carbohydrate reserve that constitutes < 1% of soybean d.m. (Karr-Lilienthal et al. 2005). The physiological levels of STA or any storage-sugar *per se* could be used as functional read-out to monitor the course of seed-germination event (Manoharlal and Saiprasad 2019). Our quantitative analysis revealed a significant decline in STA level in SSF in contrast to SPF (Fig. 4a), indicating the expected utilization of storage sugar in germinating soybean sprouts. Soybean seeds SS constitute up to 17% of total carbohydrates (Hymowitz and Collins 1974). Within SS, SUC (41.3–67.5%) and RFOs i.e. STAC (12.1–35.2%) and RAF (5.2–15.8%) are the major constituents that corresponds to 1.5–10.2%, 1.4–6.7% and 0.1–2.1% of the total d.m. (Hymowitz and Collins 1974, Yazdi-Samadi et al. 1977, Hou et al. 2009). SS also includes the traces of GLU, GAL, FRU, VER, arabinose, mannose, pinitol and myo-inositol (Yazdi-Samadi et al. 1977, Eldridge et al. 1979). A significant decline in total SS level has been observed for SSF as compared to SPF (Fig. 4b). On the contrary, RS level showed a significant increment in SSF as compared to SPF (Fig. 4c). The observed changes in STA, SS and RS levels can be explained by the fact that there is cumulative degradation of STA and SS into simple sugars, thereby providing a supply of substrate for energy/respiration to developing embryos during soybean germination. Regarding mono-saccharides, a significant increment in GLU, in concomitant with slight

increment in GAL levels was observed in SSF as compared to SPF (Fig. 4d). Notably, we were not able to detect FRU levels in either of SPF or SSF (Fig. 4d). Regarding soybean seeds oligo-saccharides which comprises ~ 5% of soybean d.m., primarily consists of SUC (β-D-fructofuranoside) and RFOs (galacto-oligosaccharides) (Karr-Lilienthal et al. 2005). We observed a relative and significant decline in SUC and total RFOs content for SSF in contrast to SPF (Fig. 4e). Within RFOs, two major RFOs component in soybean seeds are RAF and STA that constitutes 0.1–2.1% and 1.4–6.7% of total d.m., respectively (Kuo et al. 1988). We observed a significant decline in tested RFOs component (*viz.* VER, RAF and STA) of in SSF (Fig. 4e). This observation could also be complimented by fact there is an enhanced endogenous levels of α-galactosidase (*AGA*) / melibiase (α-D-galactoside galactohydrolase) during soybean germination that resulted in a potent hydrolysis of indigestible and anti-nutritional RFOs into digestible and nutritional di-saccharides i.e. SUC, which in turn upon its further hydrolysis to mono-saccharides (*viz.* GLU) could be used as readily available carbon/energy-source for soybean germination (Mostafa et al. 1987). Likewise, molecular characterization of α-GAL family member (*OmAGAL2*) in *O. minor* revealed its involvement in hydrolysis of its storage polysaccharide, planteose [α-D-galactopyranosyl-(1→6)-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside] after the perception of SLs, which in turn provides the essential hexoses for germination (Wakabayashi et al. 2015). Since RFOs are α-1,6-galactosyl extensions of SUC (Suc-Gal_n) and SUC levels showed a significant decline in SSF, henceforth a concomitant decline in RFOs levels was always anticipated. Other way around, it can be also argued that SUC being a by-product of RFOs hydrolysis, its levels should have shown a marked increase in SSF. However, this hypothesis can be easily refuted by the fact that during soybean germination, SUC catabolic enzyme *viz.* invertase (*Inv*, β-D-fructofuranosidase) and a reversible-acting SUC anabolic enzyme *viz.* SUC synthase (sucrose-UDP glucosyltransferase) showed a characteristic up-regulated expression/functional activity (Manoharlal and Saiprasad 2019). Thereby, the observed decline in SUC content of SSF could be attributed mainly due to its enhanced breakdown in concomitant with its reduced biogenesis. The validity of these identified sugars (i.e. GLU, GAL, FRU, SUC, VER, RAF and STAC) as a potential allelochemical(s) were evaluated by testing the empirical concentration (10 mM) of each sugar as a pre-conditioning media on *in vitro* germination of *O. cernua*. Our results demonstrated that GLU and GAL cause a significant to marginal improvement, while FRU and SUC causes either not detectable (*n.d.*) to marginal improvement in CGR of *O. cernua* (Fig. 6b). On the contrary, RAF, STAC and VER cause a significant decline in CGR of *O. cernua* (Fig. 6b). All these observations were further corroborated by use of SPE_{50} and SSE_{50} as a pre-conditioning media that were prepared from SPF and SSF pre-treated with an enzyme mix harbouring α-GAL + INV (Fig. 6c). Overall, these results established a direct-correlation between soybean physiological levels of GLU and GAL and broomrape seed-germination. Conversely, physiological levels of soybean oligo-saccharides (*viz.* SUC, RAF, STAC and VER) demonstrated an inverse-correlation with broomrape seed-germination (Fig. 6c and *inset*).

Next, we ought to evaluate the physiological levels of soybean IFs and its impact as potential allelochemical on broomrape seed-germination. Leguminous flavonoids, being recognized as a key allelochemical, mediates the plant-microbial interactions (e.g. nitrogen-fixing rhizobium, arbuscular mycorrhizal fungi and *Fusarium* sp.) (Weston and Mathesius 2013). IFs, a sub-class of natural bio-

flavonoids and non-steroidal estrogen mimics (*a.k.a.* phytoestrogen), constituting a large group of plant secondary-metabolites has been also known to possess allelopathic activity (Guo et al. 2011, Sugiyama 2019). The first example of IFs (uncinanone A, B and C) having potential allelopathic effect on the parasitic-plant, *S. hermonthica*, was extracted from the aqueous root-exudate of cattle forage legume, Silver-leaf (*Desmodium uncinatum*) (Tsanuo et al. 2003). In a separate study, the methanolic-extract of pea (*P. sativum*) shoots has been shown to possesses an IF (i.e. formononetin, 7-hydroxy-4´-methoxyisoflavone) derived allelochemical, pisatin 1 (3-methoxy-6a-hydroxy-8,9-methylenedioxypterocarpan), which indeed was the first purified and chemically identified phytoalexin (Perrin and Bottomley 1962, VanEtten and Pueppke 1976). Regarding soybean, it has been considered as richest natural source (up to 3 mg/g on d.w. basis) of IFs (Coward et al. 1993). IFs from soybean roots-exudates has been demonstrated to maintain its rhizosphere homeostasis (Sugiyama et al. 2017). Recently, soyasaponins (triterpenoid saponins) with an allelochemical impact has been fractionated from soybean roots-exudates (Tsuno et al. 2018). Soyasaponins are composed primarily of soyasapogenol, an aglycone Ifs (AIFs) and oligo-saccharide moieties (Tsuno et al. 2018). Notably, in soybean as well as other plants, major and parental IFs are DAI, GLY and GEN, present in 2 forms: free (aglycones) and conjugated [glycosides: glucosides (β-glycosides), acetyl-glucosides ester (6'-o-acetylglycosides) and malonyl-glucosides ester (6'-o-malonylglycosides)], wherein former is the most bioactive and bio-available form (Lee et al. 2005). For instance, the strong inhibitory allelopathic activity of *Pueraria montana* (*a.k.a.* Kudzu), a leguminous naturally grown plant mainly in Japan and East Asia, has been attributed primarily to DIA (Kobayashi et al. 2021). In specific, GEN as a signal-flavonoid allelochemical has been identified from the roots-exudates of white lupin (*Lupinus lutens* L.) and soybean that plays an important role in legume-rhizobium symbiosis (Kneer et al. 1999, Sugiyama et al. 2007). Likewise, tricin, an allelochemical discovered from rice root-exudates, is an AIF (5,7,4'-trihydroxy-3',5'-dimethoxyflavone) exerts its allelopathic impact on paddy-weeds and soil-microbes (Kato-Noguchi and Ino 2003, Kong et al. 2007). Considering these facts, henceforth we specifically evaluated the physiological levels of AIFs in SPF and SSF. Our results demonstrated a relative enhanced level of total IFCs in SSF over SPF, wherein the total as well as individual AIF component (DAI, GLY and GEN) showed a significant increment (Fig. 5). The functional validity of DAI, GLY and GEN as a potential soybean allelochemical(s) were verified by testing the impact of empirical concentration (50 µM) of their purified version as a pre-conditioning media on *in vitro* germination of *O. cernua*. Interestingly, our results demonstrated that only GEN causes a significant enhancement, whereas DAI and GLY imposes a very marginal impact on CGR of *O. cernua* (Fig. 6d). The allelopathic mode of action of GEN could be attributed to its physiological role as a DNA demethylating-agent. GEN has been identified as universal DNA demethylating agent in plants and mammals that modulate the DNA methyltransferases (DNMTs) activity and restores the expression of DNA methylation silenced genes (Arase et al. 2012, Xie et al. 2014). The physiological implication of DNA de-methylating agent (*e.g.* 5-azacytidine) on *P. ramosa* germination has been positively-linked with its global as well as gene-specific DNA demethylation status (Lechat et al. 2015). Notably, to a certain extent, the fate and allelopathic activity of released plant secondary-metabolites is determined by soil-microbial dynamics (Inderjit 2005). For example, the decomposing-tissues of *Brassicaceae* plants release a characteristic secondary-metabolite,

glucosinolates, which upon conversion to isothiocyante by myrosinase acts as a toxic allelochemical to its surrounding pests (Borek et al. 1998). Likewise, wheat seedlings synthesize 2-(2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one)-β-D-glucopyranose (DIMBOA-Glc), which upon its leaching and bio-transformation (by soil-microbes) to its AIF form, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and subsequently to 6-methoxy-benzoxazolin-2-one (MBOA) exerts its broad-spectrum allelopathic impact (Macías et al. 2004, Understrup et al. 2005). Thereby, we argue that the observed non-allelopathic impact of DAI and GLY could be either due to lack of inherent allelochemical-properties or possibly due to acquired allelopathic properties only after its bioconversion to certain active forms. However, all these speculations need further and deeper investigations to make any substantial claims. Of note, all the exogenously tested reagents partially mimic the broomrape seed-germination response to SPE and/ SSE when used alone, indicating that there is either physiological synergism or a simple cumulative impact of identified bioactives of soybean seeds- and sprout-extract. A possible involvement of other unidentified bioactive(s) / allelochemical(s) from soybean-seeds and -sprouts also can't be ruled out at this stage.

Conclusion and Future perspectives

Soybean, being a well-recognised trap-crop and having allelopathic potential, offers a 'Green' method of parasitic-weed control. Our present study demonstrated differential germination behaviour of broomrape towards soybean seed- and sprouts-extract. Comparative biochemical evaluation coupled with subsequent validations revealed GA_4, ABA, sugars (GLU, GAL, SUC and RFOs) and GEN as potential soybean allelochemicals for broomrape seed-germination. Going forward, the practical execution of these identified soybean allelochemicals as natural bio-herbicide model (in terms of specificity, efficacy, cost-effectiveness, pesticide-formulations, safety and usage) forms an integral part of our future broomrape infestation control-strategies in agronomically important crops.

Abbreviations

PGRs Plant growth regulators

GAs Gibberellins

ABA Abscisic acid

RFOs Raffinose family oligosaccharides

IFC Isoflavone content

Declarations

Author(s) contribution

RM designed, executed the experiments and drafted the manuscript. GVSS guided and facilitated the research.

Conflict of interest statement

Both the authors have read and approved the manuscript. The authors firmly affirm that there was no conflict of interest. The content of this manuscript does not necessarily reflect the views or policies of ITC Limited, ITC Life Sciences and Technology Centre (ITC-LSTC).

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Figures

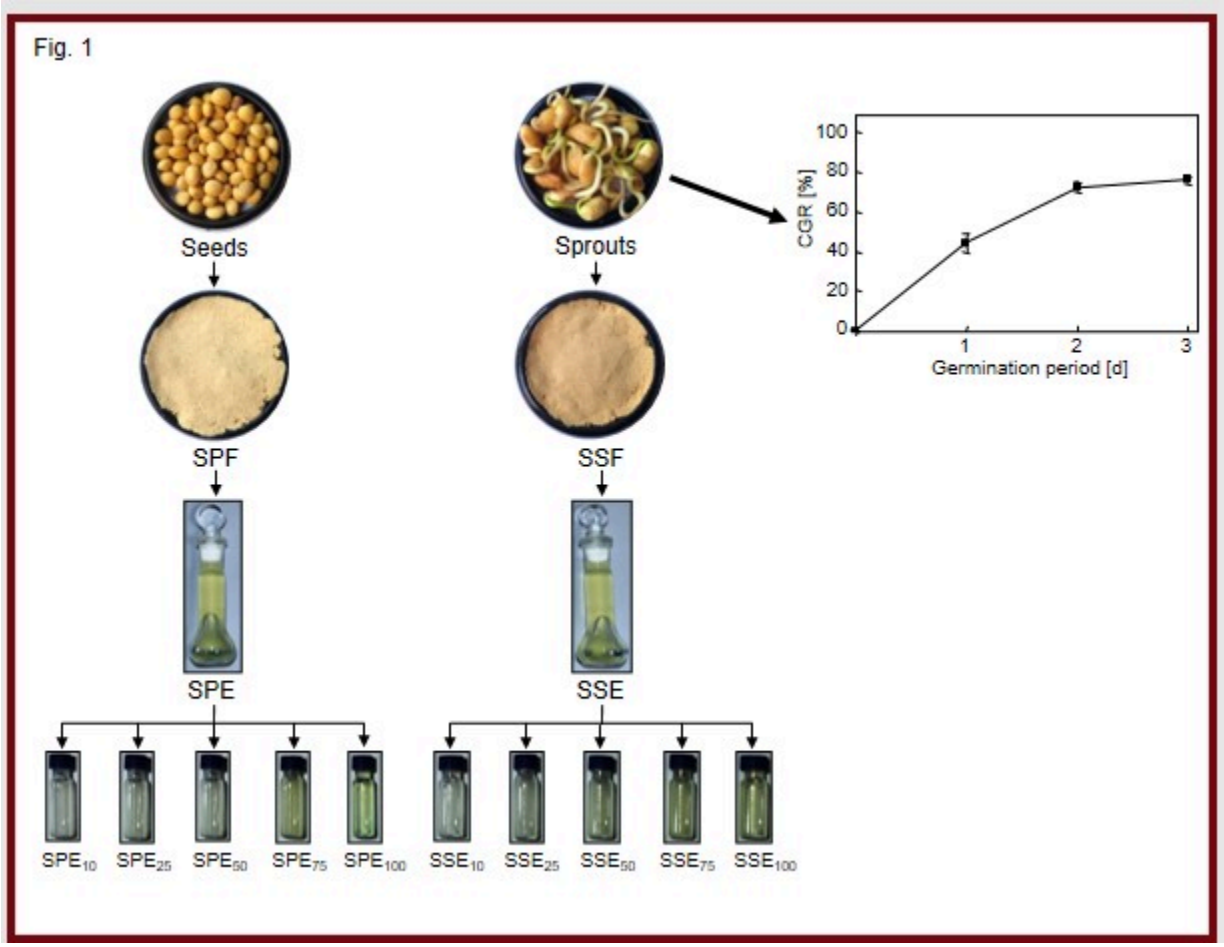

Figure 1

Soybean-sprouting, flours and -extracts preparation. Flow-chart depicts the soybean-seeds and -sprouts, correspondingly derived flours (*viz.* SPF and SSF), extracts (*viz.* SPE and SSE) and its various tested concentration (10%, 25%, 50%, 75% and 100%). *Inset* depicts the soybean seeds CGR (%) during the course of test-period. SPE_{10}, SPE_{25}, SPE_{50}, SPE_{75}, SPE_{100} and SSE_{10}, SSE_{25}, SSE_{50}, SSE_{75}, SSE_{100} corresponds to ethanolic-extract at a concentration (*v/v*) of 10%, 25%, 50%, 75% and 100% for SPE and SSE, respectively. d - day(s), SPF - soybean seeds-flour, SSF - soybean sprouts-flour, SPE - soybean seeds-extract, SSE - soybean sprouts-extract and CGR - cumulative germination-rate.

Fig. 2

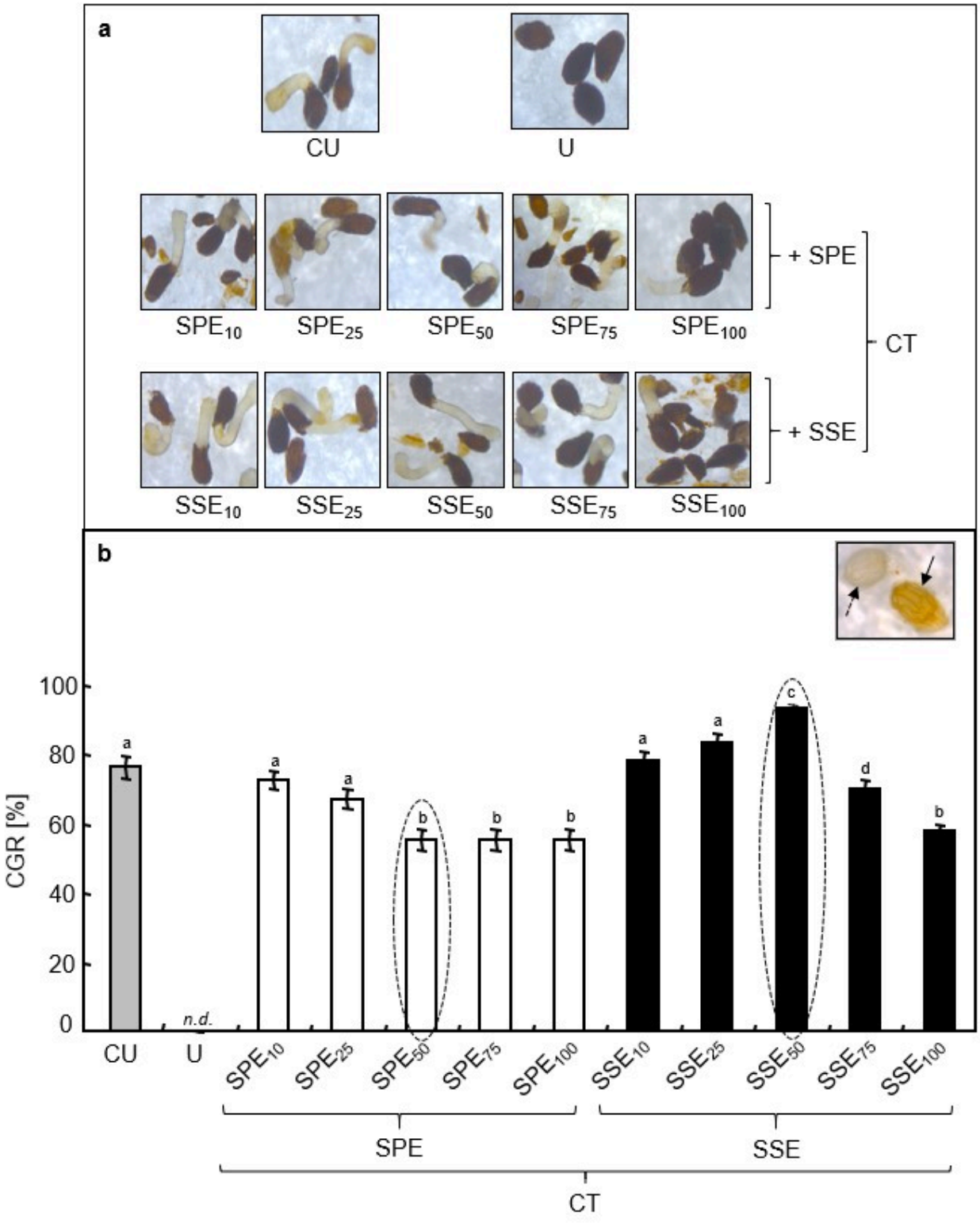

Figure 2

Impact of SPE_{50} and SSE_{50} on *in vitro* seed-germination of broomrape. Following pre-conditioning with distilled water (CU) over a test-period of 7 days, nodding broomrape seeds were subjected to stimulation with GR_{24} (10 mg/L) under prescribed assay-conditions. As a measure of pre-conditioning impact, U seeds were also stimulated with GR_{24}. Likewise, broomrape seeds were also pre-conditioned with the

indicated concentration of SPE and SSE (i.e. CT), followed by stimulation with GR_{24}. (a) Representative micrographs depicts the *O. cernua* seeds / germlings at the end of experiment. (b) In all cases, CGR (%) was measured at end of test-period, base-corrected with broomrape seed-viability (%) and plotted as bar-graph. The *marked bars* indicates the optimum suppressive- and stimulating-effect on *O. cernua* CGR at SPE_{50} and $SSE_{50,}$ respectively. *Inset* depicts the representative TTC stained broomrape seeds, wherein a non-viable seed was visible without any staining (*dotted-arrow*), alongside a yellow/orange stained viable seed (*solid-arrow*). SPE_{10}, SPE_{25}, SPE_{50}, SPE_{75}, SPE_{100} and SSE_{10}, SSE_{25}, SSE_{50}, SSE_{75}, SSE_{100} corresponds to ethanolic-extract at a concentration (*v/v*) of 10%, 25%, 50%, 75% and 100% for SPE and SSE, respectively. Means ± SDs, *n*=3, with 50 seeds per measurement. Data marked with different letters are significantly ($p \leq 0.05$) different. TTC - 2,3,5-triphenyl tetrazolium chloride, U - Unconditioned, CU - conditioned untreated, CT - conditioned treated, CGR - cumulative germination-rate and *n.d.* - not detected.

Fig. 3

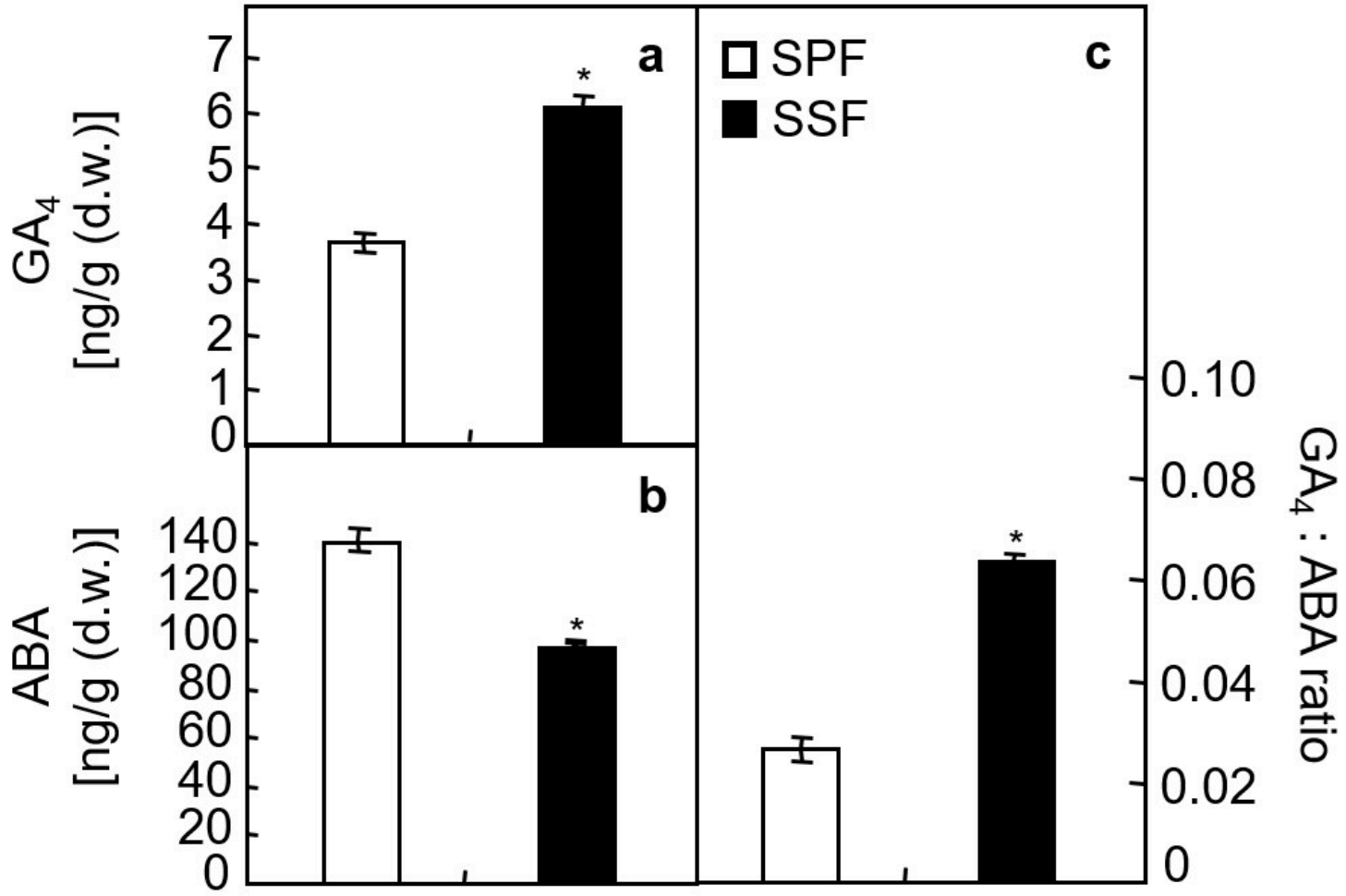

Figure 3

Measurement of soybean endogenous PGRs level. Endogenous levels of PGRs *viz.* (a) $GA_{4,}$ (b) ABA and (c) GA_4 : ABA ratio were determined from SPF and SSF. Means ± SDs, *n*=3. *Asterisks* indicate the

significant difference ($p \leq 0.05$) between group. PGRs - plant-growth regularors, GA_4 - gibberellic acid A_4, ABA - abscisic acid, SPF - soybean seeds-flour and SSF - soybean sprouts-flour.

Fig. 4

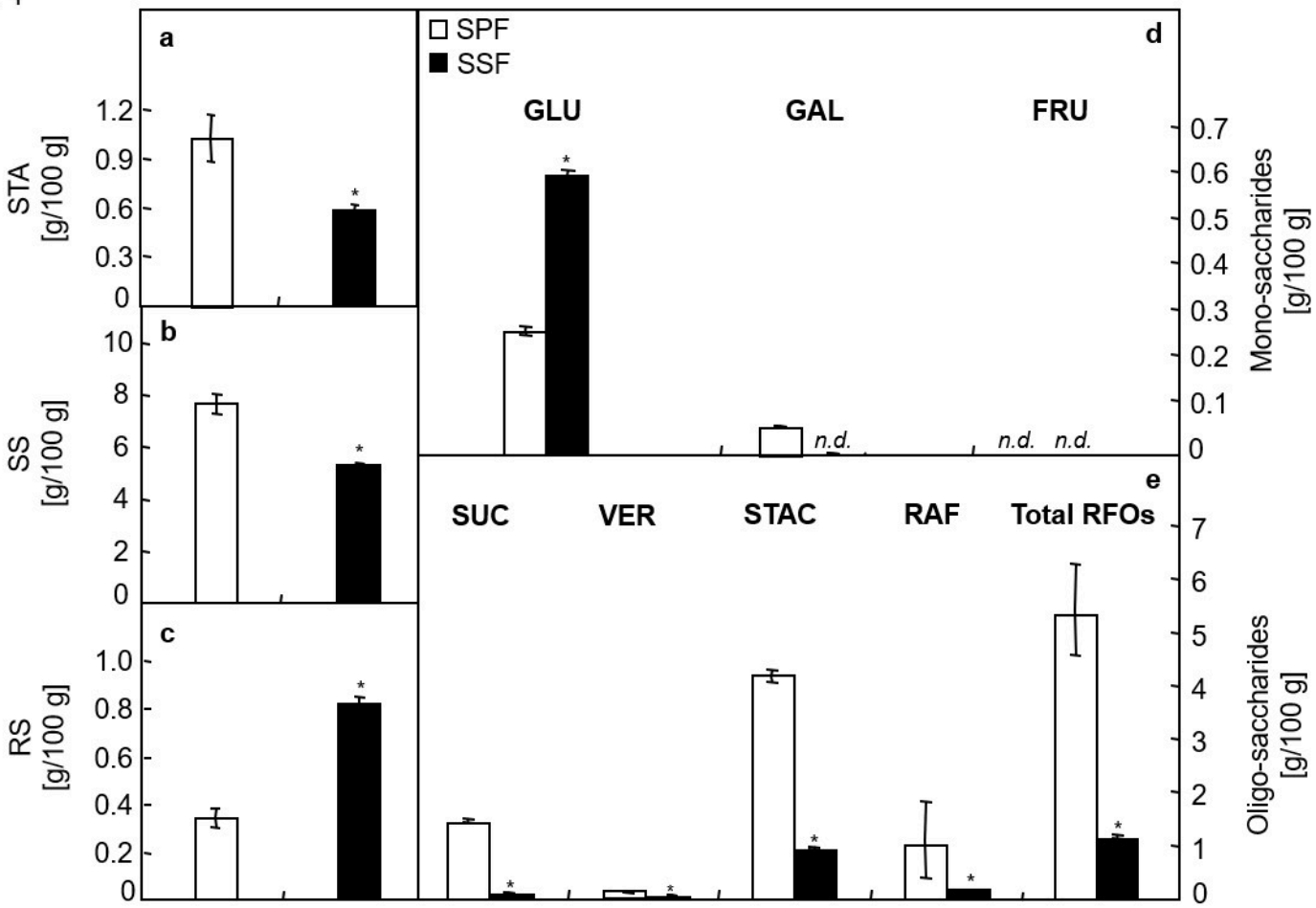

Figure 4

Measurement of soybean endogenous sugar-level. Quantitative estimation of (a) STA, (b) SS, (c) RS, (d) mono-saccharides *viz.* GLU, GAL, FRU and (e) oligo-saccharides *viz.*SUC and RFOs (i.e. RAF, STAC and VER) from SPF and SSF.Means ± SDs, $n=3$ *Asterisks* indicate the significant difference ($p \leq 0.05$) between group. STA - starch, SS - soluble-sugars, RS - reducing-sugars, GLU - glucose, GAL - galactose, FRU - fructose, SUC -sucrose, RFOs - raffinose family oligosaccharides, VER - verbascose, STAC - stachyose, RAF - raffinose, SPF - soybean seeds-flour, SSF - soybean sprouts-flour and *n.d.* - not detected.

Fig. 5

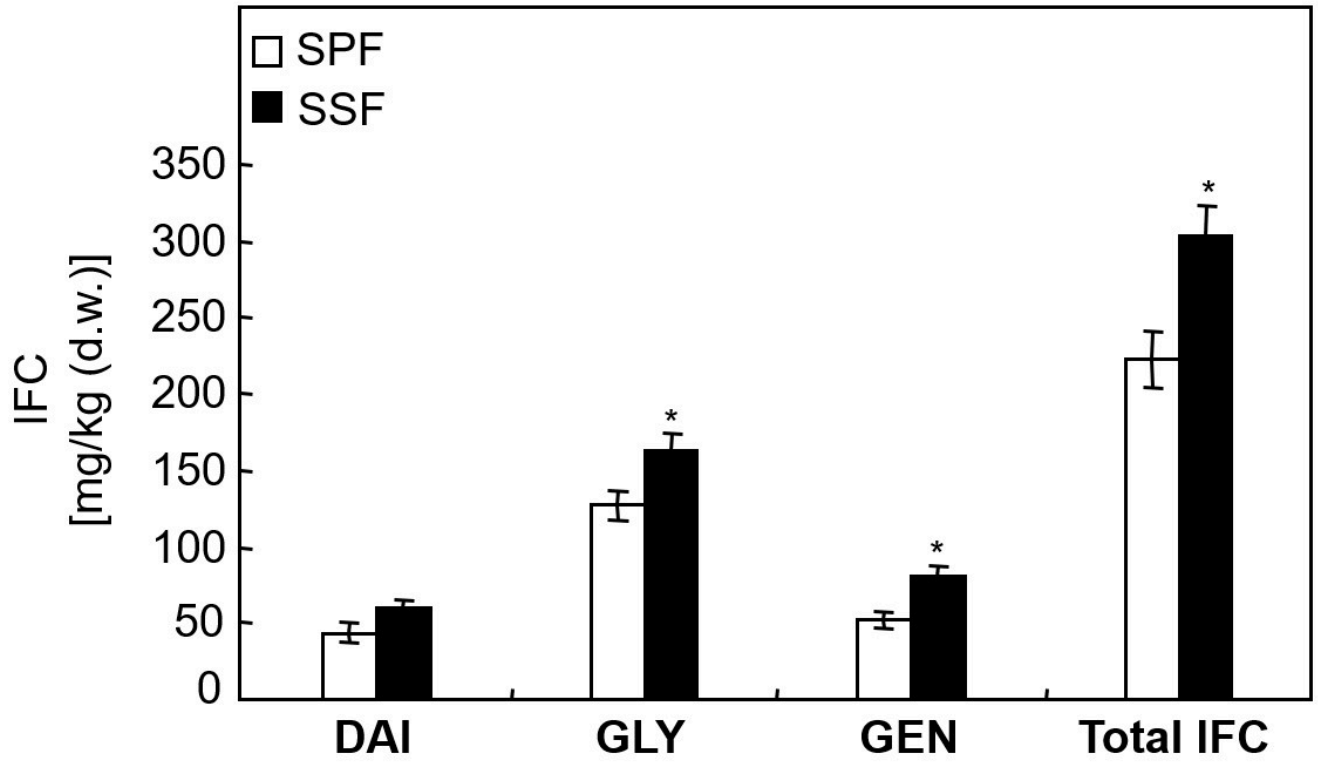

Figure 5

Measurement of soybean endogenous IFC. An HPLC based evaluation of total IFC and individual AIF bioactive (*viz.* DAI, GLY and GEN) from SPF and SSF. Means ± SDs, n=3 *Asterisks* indicate the significant difference ($p \leq 0.05$) between group. IFC - isoflavone content, AIF - aglycone isoflavone, DAI - daidzein, GLY - glycitein, GEN - genistein, SPF - soybean seeds-flour and SSF - soybean sprouts-flour.

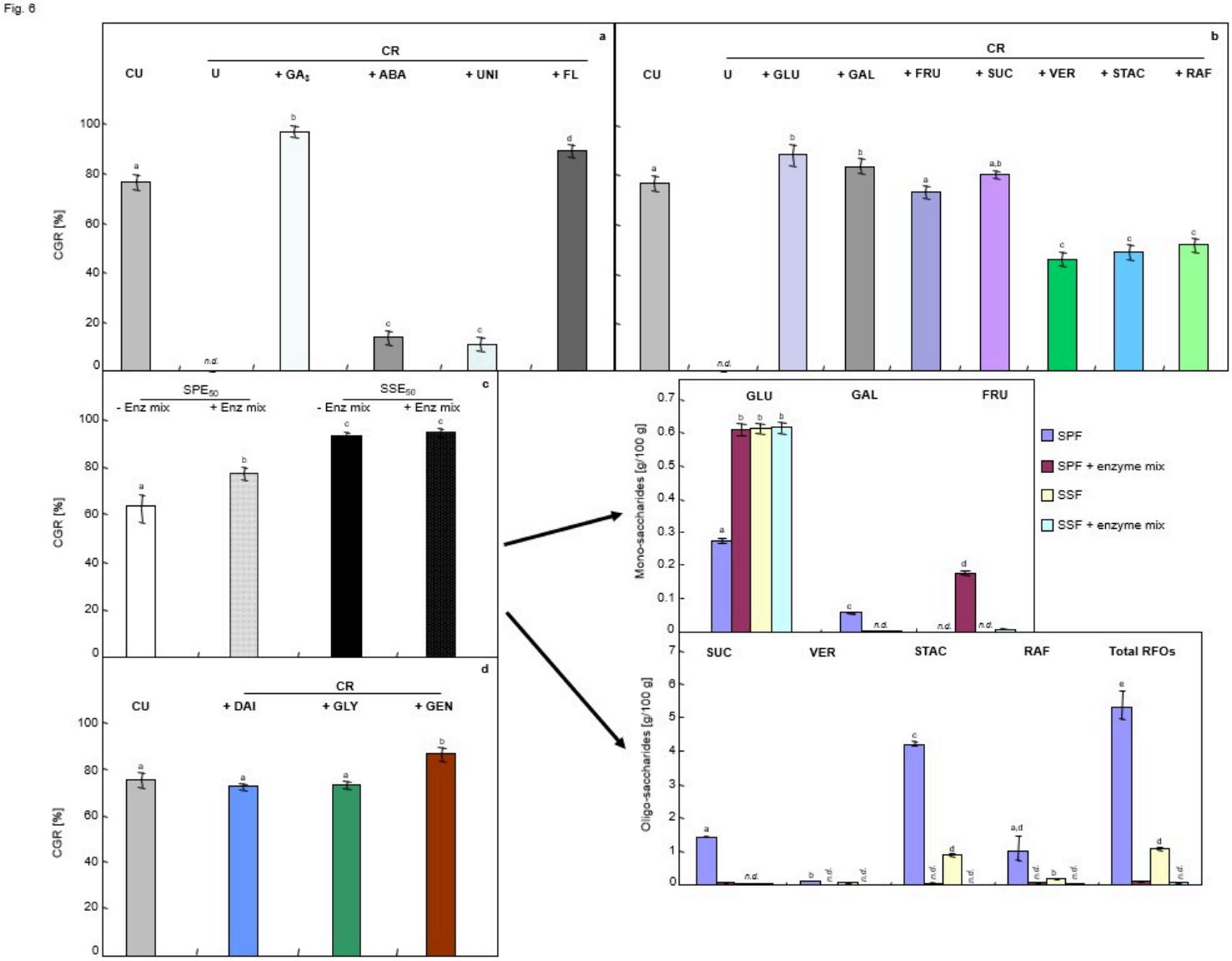

Figure 6

Impact of specific reagent(s) as a pre-conditioning media on *in vitro* seed-germination of broomrape. Broomrape seeds were pre-conditioned with either distilled water (CU) or below-mentioned specific-reagents, followed by stimulation with GR_{24} (10 mg/L) under prescribed assay-conditions. Broomrape seeds were pre-conditioned with specific reagent (CR), followed by stimulation with GR_{24} (10 mg/L) under prescribed assay-conditions. Various pre-conditioning media used were: (a) plant-growth regularors (PGRs) and its antagonists: GA_3 (25 mg/L), ABA (300 µM), UNI (0.5 mg/L) and FL (50 mg/L), (b) sugars: GLU (10 mM), GAL (10 mM), FRU (10 mM), SUC (10 mM), VER (10 mM), RAF (10 mM), STAC (10 mM) and (c) enzyme-mix (harbouring 100 α-GAL U/ml + 15 INV U/ml) pre-treated SPE_{50} and SSE_{50}. One α-GAL unit is the amount of enzyme required to liberate p-nitrophenol from the synthetic substrate p-nitrophenyl-α-D-galactopyranoside at the rate of 1 µmol/min at pH 6.5 at 37 °C. One invertase unit is the amount of enzyme required to hydrolyse the sucrose to invert sugar at the rate of 1 µmol/min at pH 4.5 at 55 °C. *Insets* depicts the comparative sugar analysis of SPF and SSF (pre- and post-enzymatic mix treatment) (d) AIFs: DAI (50 µM), GLY (50 µM) and GEN (50 µM). As a pre-conditioning control, U seeds

were also stimulated with GR_{24}. Representative micrographs depict the *O. cernua* seeds / germlings at the end of experiment (Fig. S8). In all cases, CGR (%) was measured at end of test-period, base-corrected with broomrape seed-viability (%) and plotted as bar-graph. Means ± SDs, n=3, with 50 seeds per measurement. Data marked with different letters are significantly ($p \leq 0.05$) different. U - Unconditioned, CU - conditioned untreated, CR - conditioned with specific reagent, GA_3 - gibberellin A_3, ABA - abscisic acid, UNI - uniconazole, FL - fluridone, GLU - glucose, GAL - galactose, FRU - fructose, SUC - sucrose, VER - verbascose, STAC - stachyose, RAF - raffinose, α-GAL - α-galactosidase, Inv - invertase, SPE_{50} - 50% concentrated (*v/v*) ethanolic soybean seeds-extract, SSE_{50} - 50% concentrated (*v/v*) ethanolic soybean sprouts-extract, SPF - soybean seeds-flour, SSF - soybean sprouts-flour, AIFs - aglycone isoflavones, DAI - daidzein, GLY - glycitein, GEN - genistein, CGR - cumulative germination-rate and *n.d.* - not detected.

Supplementary Files

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- SupplementaryInformation.doc
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