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RESEARCH PAPER

Nitric oxide accelerates germination via the regulation of respiration in chickpea

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Abstract

Seed germination is crucial for the plant life cycle. We investigated the role of nitric oxide (NO) in two chickpea varieties that differ in germination capacity: Kabuli, which has a low rate of germination and germinates slowly, and Desi, which shows improved germination properties. Desi produced more NO than Kabuli and had lower respiratory rates. As a result of the high respiration rates, Kabuli had higher levels of reactive oxygen species (ROS). Treatment with the NO donor *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) reduced respiration in Kabuli and decreased ROS levels, resulting in accelerated germination rates. These findings suggest that NO plays a key role in the germination of Kabuli. SNAP increased the levels of transcripts encoding enzymes involved in carbohydrate metabolism and the cell cycle. Moreover, the levels of amino acids and organic acids were increased in Kabuli as a result of SNAP treatment. ^{1}H-nuclear magnetic resonance analysis revealed that Kabuli has a higher capacity for glucose oxidation than Desi. An observed SNAP-induced increase in ^{13}C incorporation into soluble alanine may result from enhanced oxidation of exogenous [^{13}C]glucose via glycolysis and the pentose phosphate pathway. A homozygous hybrid that originated from a recombinant inbred line population of a cross between Desi and Kabuli germinated faster and had increased NO levels and a reduced accumulation of ROS compared with Kabuli. Taken together, these findings demonstrate the importance of NO in chickpea germination via the control of respiration and ROS accumulation.

Keywords: Alternative oxidase, hydrogen peroxide, nitric oxide, nitrite, reactive oxygen species, superoxide.

Introduction

To perform key physiological and biochemical processes underpinning growth, plants require energy in the form of ATP. The production of ATP via oxidative phosphorylation requires oxygen (Millar *et al.*, 2011). Oxygen also plays an important role in regenerating NAD^+ from NADH, in haem, sterol, and fatty-acid biosynthesis (Geigenberger *et al.*, 2000). Due to the lack of an efficient system for the distribution of oxygen, some tissues such as seeds, tubers, and underground roots experience hypoxia. For instance, internal oxygen concentrations in various tissues vary due to tissue size, developmental stage, and

Abbreviations: AOX, alternative oxidase; COX, cytochrome *c* oxide; DAB, 3,3′-diaminobenzidine; DAF-FM DA, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; NBT, nitroblue tetrazolium; NO, nitric oxide; ROS, reactive oxygen species; SNAP, *S*-nitroso-*N*-acetyl-D,L-penicillamine; SNP, sodium nitroprusside.

location (Borisjuk and Rolletschek, 2009). Poor availability of oxygen was reported in various plant tissues, with the levels of internal oxygen ranging from zero to 40% of air saturation (Rolletschek *et al.*, 2002). Seeds of several species, such as *Zea mays*, *Brassica napus*, *Glycine max*, *Hordeum vulgare*, *Helianthus annuus*, and *Pisum sativum*, maintain internal oxygen concentrations in the range of approx. 2–10 μM (Rolletschek *et al.*, 2003). High levels of respiration can deplete oxygen very quickly and non-bulky tissues can compensate for this oxygen loss by taking oxygen from the atmosphere. However, bulky tissues face diffusion constraints to acquire sufficient oxygen to achieve oxygen homeostasis (Borisjuk *et al.*, 2007; Gupta *et al.*, 2009).

Depletion of internal oxygen can cause hypoxia and eventually anoxia within tissues, which can cause serious problems by affecting plant metabolism (Geigenberger *et al.*, 2000). If mitochondrial respiration stops, there is fall in ATP production per mole of glucose metabolized. In order to compensate for this energy loss, plants attempt to generate energy via alternative pathways. For instance, under low oxygen availability, glycolytic fermentation and the phytoglobin–nitric oxide cycle are the energy producing pathways promoting the synthesis of products such as ethanol, lactate, alanine, γ-aminobutyric acid, succinate, and malate (Igamberdiev *et al.*, 2004, 2010; Stoimenova *et al.*, 2007; Rocha *et al.*, 2010; Wany *et al.*, 2018). Nitrite reduction to nitric oxide (NO) was also recently shown to generate energy (Vishwakarma *et al.*, 2018). Plants have various biochemical adaptations to cope with low oxygen (Fox *et al.*, 1994; Sousa and Sodek, 2002). One such adaptation is regulation of respiration (Gupta *et al.*, 2009; Gupta *et al.*, 2014). Recently it was demonstrated that plants can decrease their oxygen consumption in response to low oxygen concentrations to avoid internal anoxia (Zabalza *et al.*, 2009). Nitric oxide generated by the mitochondrial electron transport chain can decrease oxygen consumption at the frontier of anoxia (Benamer *et al.*, 2008). Moreover, plants can decrease ATP consumption by shutting down various energy requiring biochemical processes (Geigenberger *et al.*, 2000).

Seed germination is crucial for the plant life cycle. One of the essential factors is the transition of the seed from dormancy to germination. This phase requires a series of metabolic, biochemical, and molecular events to initiate germination. After water imbibition, the seed starts to germinate, producing various reactive oxygen species (ROS) such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) (Bailly, 2004; Kranner *et al.*, 2010). Moreover, oxygen deficiency can promote increased ROS production (Vergara *et al.*, 2012). If not detoxified, ROS can damage cellular structures such as membranes and promote protein degradation, thus delaying the germination process (Hu *et al.*, 2007). Recent studies have shown that NO, a gaseous free radical, plays a crucial role in seed germination (Libourel *et al.*, 2006; Zheng *et al.*, 2009; Bykova *et al.*, 2015) and mitochondrial function (Yamasaki *et al.*, 2001; Gupta *et al.*, 2017). Exogenous application of NO donor compounds such as sodium nitroprusside (SNP), nitrite, and nitrate break dormancy in Arabidopsis (Bethke *et al.*, 2004; 2006). However, how NO modulates primary metabolism to improve germination has not been completely unravelled. NO is known to inhibit mitochondrial respiration by reversible binding to cytochrome *c* oxidase (Millar and Day, 1996). This inhibition helps in improving oxidative phosphorylation (Clerc *et al.*, 2007). An interplay and balance between NO, ROS, and respiratory metabolism may be needed for the maintenance of energy status within the seed during germination. For instance, minimizing ROS levels may be required to initiate germination. NO might play a role in this mechanism of ROS detoxification by inducing the synthesis of antioxidants (Wany *et al.*, 2018). However, currently there are no reports on the mechanism of ROS detoxification during the progress of seed germination. This information would be useful for economically important crops like chickpea.

There are two main cultivars of chickpea: Desi and Kabuli. The Kabuli variety has larger seeds but a lower germination rate. This may be due to its larger size and differences in respiratory metabolism. Therefore, in the present study we addressed (i) the relationship between respiration and ROS in Desi and Kabuli, (ii) the role of NO in controlling ROS and internal oxygen to promote seed germination, and (iii) the impact of NO on the modulation of various genes and metabolites during germination. We found that the maintenance of oxygen homeostasis within the developing seed is essential to prevent hypoxia, and NO is the key regulator in this context. The Kabuli variety has reduced internal oxygen and NO and increased respiration. We found that NO supplementation in the form of *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) helps in increasing internal oxygen, reducing ROS, and improving germination in Kabuli. Further, our data showed that a recombinant inbred line (RIL) hybrid chickpea that originated in an intercross between Desi and Kabuli had improved NO content and germination capacity pointing to the importance of NO in the regulation of internal oxygen and germination.

Materials and methods

Plant material and seed germination

In the present study, seeds of chickpea were used, namely Desi chickpea (Pusa 372), Kabuli chickpea (Pusa 1088), and a homozygous intercross hybrid of Desi and Kabuli selected from a RIL mapping population (Pusa 372 × Pusa 1088). Healthy mature seeds of identical weight were first rinsed with sterile water and then treated with 0.1% $HgCl_2$ for 10 min. Seeds were rinsed five to six times with sterile water and kept soaked in autoclaved distilled water for 4 h for imbibition. Thereafter, excess water was drained out, and seeds were wrapped in wet cheesecloth and kept for germination at 23 °C (Pandey *et al.*, 2017*a*).

Germination response in presence of nitric oxide donor

To determine germination percentage, chickpea seeds were germinated in sand (25 g in a Petri dish). NO donors SNAP 500 μM, SNP 500 μM, potassium nitrite (KNO_2) 0.5 mM and distilled water as a control were added to the sand. Seeds were kept in the dark until emergence of the radicle. Germination percentage was recorded after 24, 48, and 72 h.

Respiration and internal oxygen measurements

For respiratory and internal oxygen measurements, a TBR1025 single channel oxygen sensor (World Precision Instruments, Sarasota, FL, USA) was used as previously described in Pandey *et al.* (2017*a*). For calibration, the oxygen sensor was rinsed with distilled water and then dipped into

a vial containing 5 ml of air-saturated water to establish 100% oxygen; for zero oxygen, water was bubbled with 100% nitrogen gas and the sensor then calibrated. For respiratory measurements, seeds weighing 3 g were transferred to a vial containing 5 ml of 25 mM HEPES buffer (pH 7.2). After the vial was closed, the sensor was inserted and oxygen uptake was measured. For internal oxygen measurement, the microsensor was inserted to different depths (2 and 4.5 mm) and the internal oxygen recorded.

ROS measurements

Superoxide radicals were measured using nitroblue tetrazolium (NBT) staining as described previously (Jambunathan, 2010). Hydrogen peroxide (H_2O_2) production was monitored by staining the seed cotyledon with 3,3′-diaminobenzidine (DAB) solution (Daudi and Brien, 2012). For measurements, the seed coat was removed gently using forceps and the cotyledons were dipped into 2 ml 0.1% (w/v) NBT and 1 mg ml^{-1} DAB for 12 h. Stained cotyledons were rinsed with 70% ethanol and images were taken under a bright-field stereomicroscope (Nikon AZ 100, Model AZ-LED, Japan).

Nitric oxide detection by DAF-FM DA and gas phase Griess reagent assay

For detection of NO, the seed coat was removed and cotyledons were incubated in 10 μM 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA) for 30 min in the dark at room temperature. Thin sections of cotyledons were cut using a razor blade and washed in 10 mM HEPES (pH 7.2) twice to remove the unbound dye. For control, 200 μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) potassium salt was loaded along with DAF-FM DA. Imaging was performed using a fluorescence microscope (Nikon, Eclipse 80i, Japan) using λ_{488} excitation and $\lambda_{500–530}$ emission wavelengths. Gas phase NO measurements by a gas phase Griess reagent assay were performed as described by Wany *et al.* (2018).

Quantification of nitrite and NR activity levels

Nitrite levels were quantified according to Vishwakarma *et al.* (2018). To the chickpea extract, 1% sulphanilamide and 0.1% naphthylethylene dihydrochloride was added and the mix incubated for a period of 10 min at 25 °C. The absorbance was read at 546 nm. For a standard, 0.1–10 μM KNO_2 was used. NR activity was measured according to (Planchet *et al.*, 2005).

Starch estimation

Frozen control and SNAP-treated seeds were homogenized in liquid nitrogen, mixed with 80% ethanol, and centrifuged at 10 000 *g*. The pellet was mixed with pre-chilled perchloric acid (52%, v/v) and again centrifuged for 10 min at 4 °C. Pooled supernatant was mixed with 20-fold MilliQ water; 500 μl of diluted supernatant was mixed with pre-chilled anthrone–sulphuric acid reagent (2 ml of 0.2%) and placed in a boiling-water bath for 10 min. The reaction was terminated by transferring the test tubes on ice and the absorbance was obtained at 630 nm (Hansen and Møller, 1975).

Lipid peroxidation assay

The malondialdehyde content of chickpea seed was determined according to a protocol described by Jambunathan (2010). Frozen seeds (200 mg) of SNAP-treated and control were homogenized in 4 ml of 0.1% trichloroacetic acid (TCA) solution and centrifuged at 10 000 *g* for 15 min. To 1 ml of supernatant collected in a glass test tube, 2 ml of 20% TCA and 2 ml of 0.5% thiobarbituric acid was added. Finally, the reaction mixture was vortexed and heated at 95 °C for 30 min in a water bath and then transferred to ice to stop the reaction. The absorbance was recorded at 532 and 600 nm and calculated according to Jambunathan (2010).

Mitochondrial isolation, respiratory measurements and western blot analysis

Mitochondria were isolated from seeds after 48 h of seed germination, according to (Pandey *et al.*, 2017*b*). All procedures were carried out at 4 °C. Approximately 50 g of seeds were used for isolation. The final mitochondrial suspension was dissolved in 1 ml of suspension buffer. Concentration of the isolated mitochondrial protein was determined using the Bradford protein assay (Bradford, 1976).

For measurement of oxygen consumption of mitochondria, 1 mg ml^{-1} of mitochondrial protein was added to a 1.5 ml vial containing suspension buffer, and oxygen consumption was measured using a microsensor after adding 100 μM NADH.

Mitochondrial protein was mixed with 4× sodium dodecyl sulfate (SDS) loading buffer (4× Bolt™ LDS Sample Buffer, Invitrogen) and boiled for 5 min in a water bath. For SDS-PAGE, 20 μg mitochondrial protein was loaded per well. The proteins were electrophoretically transferred to nitrocellulose membranes using the iBlot™ 2 Dry Blotting System (Invitrogen, USA). Western blot analysis was carried using anti-alternative oxidase (AOX) antibody (i.e. anti-AOX1a (1:1000 dilution, Agrisera) as the primary antibody and horseradish peroxidase-conjugated anti-rabbit IgG antibody (1: 10 000 dilutions, Bangalore GeNei™) as the secondary antibody. Blots were developed using an enhanced chemiluminescence detection system for western blots that contained a sensitive, luminol-based enhanced chemiluminescent substrate for detecting horseradish peroxidase on immunoblots (Invitrogen).

DNA laddering analysis

Genomic DNA was isolated from germinated seeds (4 and 48 h) using a kit (Plant RBC, Real Biotech Corp., China) and quantified with a NanoDrop instrument (Thermo Scientific, USA). Genomic DNA of 0.5 μg was electrophoresed in 1.2% agarose gel and visualized by ethidium bromide staining.

RNA isolation and gene expression analysis

Frozen samples of 200 mg were crushed to a fine powder in liquid nitrogen and transferred to an microcentrifuge tube containing 500 μl of extraction buffer (200 mM NaOAc pH 5.2, 1% SDS, 10 mM EDTA pH 8.0) and 500 μl of phenol (Choudhary *et al.*, 2009). Samples were mixed thoroughly and centrifuged at 14 000 *g* for 20 min at room temperature. The aqueous phase was transferred to a fresh microcentrifuge tube and extracted twice with phenol:chloroform (1:1). For RNA precipitation, 0.3 volume of 10 M LiCl was added to the aqueous layer, and kept overnight at −80 °C. The RNA pellet was obtained by centrifugation at 10 000 *g* for 10 min at 4 °C. Pellet was washed twice with 2.5 M LiCl and once with 70% ethanol. After air-drying, the pellet was dissolved in diethyl pyrocarbonate-treated ddH_2O and the concentration was quantified using a NanoDrop instrument (Thermo Scientific, USA). This RNA was treated with RQ1 RNase-free DNase I (Promega, Madison, WI, USA). Total RNA of 2 μg was utilized for synthesis of cDNA using the high capacity cDNA Reverse Transcription Kit according to the manufacturer's protocol (Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit). The PCR was set in a 384-well plate using SYBR® Green qPCRMaster Mix (Agilent). The primers used for gene-specific expression in RT-PCR are listed in Supplementary Table S1 at *JXB* online. Initial denaturation was at 95 °C for 1 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 58–61 °C for 30 s. The relative expression values were calculated using elongation factor α as an internal standard (Garg *et al.*, 2010).

Methanol soluble metabolite extraction

To the lyophilized powder of chickpea seeds (100 mg), 1 ml of methanol was added followed by heating at 70 °C for 5 min. The extracts were centrifuged at 13 000 *g* (10 min, 4 °C) to separate the methanol-soluble metabolites from insoluble pellets. Supernatant (100 μl) containing the methanol-soluble metabolites was collected into fresh tube and dried by vacuum-drier at 40 °C for 1–2 h using a speed-vacuum (Labconco, USA).

Protein hydrolysis

The insoluble pellets obtained after methanol extraction were acid hydrolysed by suspending in 500 µl 6 M HCl and incubating at 100 °C on a heat block for 20 h (Antoniewicz *et al.*, 2007). The hydrolysate (made up to ~200 µl with water) was centrifuged at 13 000 *g* for 10 min to separate the amino acid supernatant from insoluble pellets; 100 µl of supernatant from each replicate was collected into fresh microcentrifuge tube and was vacuum-dried for 4 h in a speed-vacuum system (Labconco, USA). The dried hydrolysates were subjected to derivatization and GC-MS.

Metabolite extraction, GC-MS analysis

Frozen seeds (100 mg) were crushed to fine powder in liquid nitrogen to which 1400 µl of 100% methanol (HPLC grade, Sigma-Aldrich) was added and vortexed for 30 s followed by addition of 60 µl of ribitol (0.2 mg ml^{-1}) as an internal standard (Lisec *et al.*, 2006). This sample mixture was heated at 70 °C for 10 min in a thermomixer at 1000 rpm. Samples were centrifuged at 10 000 *g* for 15 min at 4 °C, supernatant was collected and to this 750 µl chloroform (HPLC grade, Sigma-Aldrich) and 1500 µl Milli Q water was added and the mix vortexed for 30 s. Centrifugation of this mixture at 5000 rpm and 4 °C for 10 min leads to formation of two layers; the upper polar phase was collected and dried in a speed vacuum. The residues obtained after evaporation were re-dissolved in 60 µl methoxyamination reagent (20 mg ml^{-1} methoxyamine hydrochloride in pyridine) and kept in a thermomixer for 2 h at 37 °C. To this 70 µl of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) reagent was added with fatty acid methyl esters (FAME mix C4–C24, Sigma-Aldrich) as retention index markers; they were mixed and subsequently incubated in a thermomixer at 37 °C for 30 min (Lisec *et al.*, 2006). The eluted metabolites were analysed by GC-MS (QP-2010, Shimadzu, Japan). The metabolites were identified by their mass fragmentation, compared with the National Institute of Standards and Technology (NIST; MD, USA) library, and quantified using ribitol as an internal standard (Kumari and Parida, 2018; Tanna *et al.*, 2018). The baseline correction of recorded GC-MS spectra was done using Metalign software (Lommen, 2009). Peaks of baselined spectra were identified using chemstation software based on a MS search from the NIST library (Golm database) and the amino acid peak integrals were also obtained using the chemstation (Agilent) software. All the amino acid peaks were identified based on the *m/z* of different fragments, their elution times, and hits against the library. These were tabulated for further natural isotope correction using Isocor software (Millard *et al.*, 2012). For fatty acid profiling, total lipid extraction and fatty acid profiling was performed as described by Patel *et al.* (2018). Approximately 500 mg of frozen seed samples was used for the analysis. For identification of proteinogenic amino acids, 100 µl dried protein hydrolysates were subjected to tertbutyldimethylsilyl derivatization and subsequent GC-MS analysis. To each dried extract, 30 µl of pyridine was added and the tubes were incubated at 37 °C for 30 min on a thermomixer set at 900 rpm. Then, to each tube 50 µl of *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MtBSTFA) + 1% *N*-methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide (t-BDMCS) + 1% *tert*-butyldimethylchlorosilane (Sigma-Aldrich) was added followed by incubation at 60 °C for 30 min in a thermomixer at 900 rpm. Subsequently, samples were centrifuged at 13 000 *g* for 10 min and the supernatant was transferred to 200 µl glass vial inserts and subjected to GC-MS.

Glucose labelling experiments

Seeds were incubated in a six-well Petri plate containing sterilized water with 0.1% (w/v) unlabelled glucose (i.e. [^{12}C]glucose, 40% [$^{13}C_6$]glucose (i.e. uniformly labelled with ^{13}C in all carbon positions) or a combination of 60% [1-^{13}C]glucose + 40% [$^{13}C_6$]glucose (i.e. ^{13}C labelled at C_1 (positionally labelled) along with uniform labelling with ^{13}C in all carbon positions). The incubated seeds and media were harvested at 0, 24 and 48 h. The supernatant was stored at −20 °C and seeds were ground into fine powder using liquid nitrogen and lyophilized. The metabolites were extracted and analysed for subsequent analysis of ^{13}C label incorporation using GC-MS.

^{1}H-nuclear magnetic resonance analysis of culture filtrates

To 200 µl of medium, 400 µl of D_2O (with 0.1% 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) as an internal standard) was added. The mixture was subjected to ^{1}H-nuclear magnetic resonance (NMR) for data acquisition. The spectra were subjected to 32 scans requiring 10 min acquisition time with the following parameters: pulse width, 11.6 µs; relaxation delay, 5 s. A presaturation sequence was used to suppress the residual H_2O signal with low power selective irradiation at the H_2O frequency during the recycle delay. The resulting spectra were manually phased, baseline corrected, and calibrated to DSS at 0.0 ppm, using ECX NMR (version 3.5, JEOL). The integrals of peaks corresponding to glucose were obtained from samples at different time points and compared with controls to give the consumption of glucose throughout the feeding experiment.

Results

Kabuli chickpea has a reduced and delayed rate of germination relative to Desi chickpea

The Desi (microsperma) and Kabuli (macrosperma) varieties of chickpea have a different size, texture, and colour. They also show a difference in storage carbohydrates, proteins, and lipids. In addition, they also give different yields. Hence, we first compared germination rates of the Desi and Kabuli varieties of chickpea. We chose commonly grown commercially available Desi 372 and Kabuli chickpea 1088. After imbibition for 4 h in water, the seeds were observed for germination capacity at 24, 48, and 72 h. Interestingly, Desi germinated more rapidly than Kabuli. Almost all seeds of the Desi variety germinated within 24–48 h whereas only 30% of Kabuli chickpea seeds germinated during this time (Fig. 1A, B). Due to the prolonged period of germination, Kabuli variety seeds were prone to infection during germination (data not shown). Hence, we further investigated the underlying mechanism of slower germination in the Kabuli variety in comparison with the Desi variety.

Kabuli has a higher respiratory rate and lower internal oxygen relative to Desi

We sought to investigate the mechanism of the slower germination rate in the Kabuli variety. Increased metabolic activity accompanied by increase respiratory rate occurs during germination. Increased energy production is essential for defending against cell wall resistance in the endosperm (Geigenberger, 2003). A difference in respiratory metabolism could be one of the reasons for the observed difference in germination potential of the two varieties (Fig. 1). Hence, first we measured the respiratory rate. For this purpose, seeds weighing 3 g were placed in a closed 5 ml vial in a buffer and the respiratory rate measured using an oxygen sensor. The Desi variety showed a respiratory rate of 107±7.5 µmol g FW^{-1} h^{-1} and the Kabuli variety showed a rate of 160±21 µmol g FW^{-1} h^{-1} at 24 h. At 48 h Desi showed a similar respiratory rate of 108±8 µmol g FW^{-1} h^{-1}, whereas Kabuli showed a higher rate of 211±20 µmol g FW^{-1} h^{-1}, suggesting that the Kabuli variety respires at a higher rate than the Desi variety. In Desi at 24 and 48 h there was no significant difference in respiratory rate whereas in Kabuli the rate was higher at 48 than at 24 h (Fig. 2).

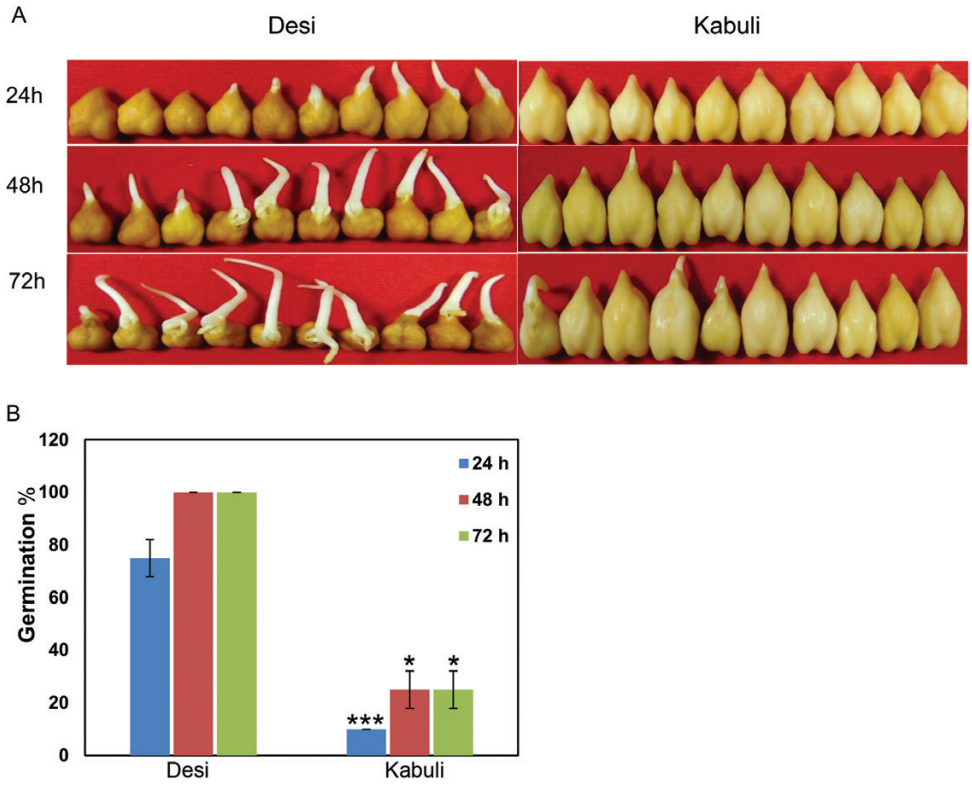


Fig. 1. (A) Germination of Desi and Kabuli chickpea at 24, 48, and 72 h post-imbibition. Image is representative of four independent experiments. (B) Germination percentage of Desi and Kabuli chickpea at 24, 48, and 72 h post-imbibition. Values are mean ±SE (n=3). Asterisks indicate significant difference between Desi and Kabuli at different time intervals; *P<0.05, ***P<0.0001, t-test.

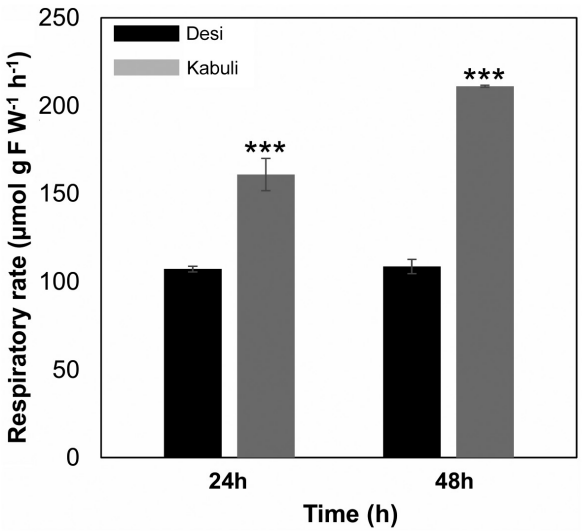


Fig. 2. Respiratory rate of Desi and Kabuli seeds at 24 and 48 h post-imbibition. Four to five seeds weighing in total 3 g were used for measuring oxygen consumption rates. Values represent mean of three replicates (n=3, ±SE). ***P<0.0001 between Desi and Kabuli at different time intervals, t-test.

Increased respiratory rate may affect internal oxygen, which may become more important in seeds that are different in size. Hence, we measured internal oxygen at 24 and 48 h by inserting an oxygen sensor into seeds at different seed depths (Fig. 3). It was found that Desi had an internal oxygen score of 30% while Kabuli had 13% internal oxygen at 2 mm depth; in the middle of the seed (4.5 mm depth) the internal oxygen was 1.7±1.8% in Desi and 0.84±0.5% in Kabuli chickpea at 24 h. Internal oxygen was further reduced at 48 h, where Desi chickpea showed 19±2.3% and Kabuli chickpea showed 7.4±2.9% at 2 mm depth. At 4.5 mm depth at 48 h Desi chickpea had 3.6±0.5% internal oxygen whereas Kabuli chickpea had 0.26±0.26%. Taken together these results suggest a negative correlation between respiration and size of the seeds for the internal oxygen concentration (compare Figs 2 and 3B).

Decreased internal oxygen impacts ROS levels

Low oxygen condition can increase ROS levels (Vergara *et al.*, 2012), and hence a decrease in internal oxygen could impact ROS, which may have an impact on seed germination (Gidrol *et al.*, 1994). Hence, we checked O_2^- levels by NBT staining (Fig. 4A). At 24 and 48 h there was increased O_2^- in both Desi and Kabuli chickpea, but in comparison with Desi chickpea, the Kabuli chickpea displayed slightly higher levels of O_2^-. At 72 h O_2^- levels were reduced in both varieties, but Kabuli chickpea displayed slightly reduced levels of O_2^- compared with Desi. Examination of H_2O_2 levels (Fig. 4B) revealed that at 24 h post-germination there was a higher H_2O_2 level in Kabuli in comparison with Desi chickpea. At 48 and 72 h there was a decrease in H_2O_2 in both varieties, but Kabuli chickpea displayed higher H_2O_2 than Desi chickpea. Taken together these results suggest a negative correlation between internal oxygen and ROS (compare Figs 3 and 4).

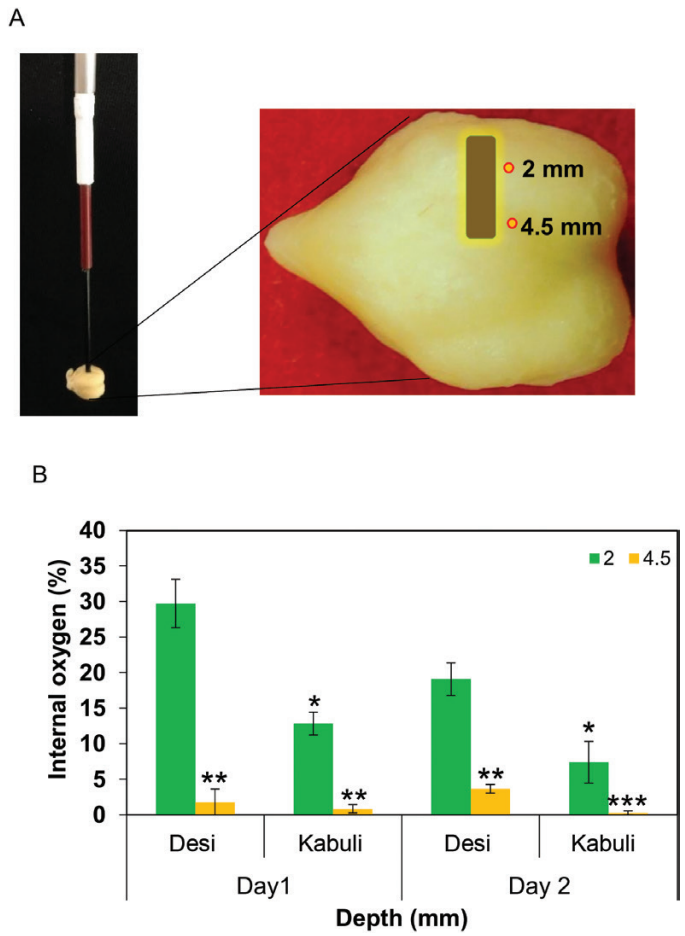


Fig. 3. Measurement of internal O_2 concentration. (A) Image of oxygen sensor incision into chickpea seed for measurement of internal oxygen levels; inset shows the areas in chickpea seed where oxygen measurements were taken. Red circle indicates depth of seed. (B) Internal oxygen levels in both varieties of chickpea a two different depths at 24 and 48 h post-imbibition. Values indicate oxygen concentration as percentage of air saturation. Values represent means of three replicates (n=3, ±SE). Significance was determined by t-test; *P<0.05, **P<0.005, ***P<0.0001 considering Desi (2 mm depth) on day 1 and day 2 as control.

Differential nitric oxide levels reflect a difference in respiration

The difference in ROS and respiratory rates might also be due to a difference in NO levels. Hence, we checked NO levels by using a gas phase Griess reagent assay. We found increased NO levels in Desi chickpea in comparison with Kabuli chickpea (Fig. 5A). We also measured NO by DAF-FM DA in imbibed seeds. DAF-FM DA fluorescence was lower in Kabuli chickpea than in Desi chickpea (Fig. 5B).

Nitric oxide treatment leads to increased germination potential

NO is known to regulate respiration via inhibition of cytochrome c oxidase (COX) (Brown, 1999). This inhibition may help in reducing the respiratory rate and hence increasing internal oxygen. Therefore, we sought to check the germination potential of these seeds in response to the NO donors SNAP, SNP, and KNO_2. Seeds were treated and checked for germination at 48 h. Strikingly SNAP and KNO_2 accelerated germination up to 70% at 48 and 72 h in Kabuli chickpea, which germinated at a slow rate with only up to 30% germination in water (Fig. 6), suggesting a potential role for NO in inducing germination in Kabuli.

NO treatment caused a decrease in respiration and ROS and an increase in internal oxygen of Kabuli chickpea

An increase in the germination potential of Kabuli chickpea by NO from the NO donor SNAP is probably due to regulation of respiration by this redox-active small molecule, which can play a role in lowering respiration in order to prevent tissue anoxia. Hence, we checked respiration in the presence or absence of the NO donor SNAP. Kabuli chickpea respired faster than Desi (this effect is most likely due to different NO levels Fig. 5), but the presence of SNAP caused a decrease in oxygen consumption (Fig. 7A), suggesting that NO can indeed regulate respiration in Kabuli. The decreased respiration in Kabuli can increase internal oxygen, and hence we measured internal oxygen in the presence or absence of SNAP. Internal oxygen level in Desi was elevated from 21% to 27%, whereas in Kabuli the level was increased from 10% to 22% (Fig. 7B), suggesting that SNAP can indeed increase internal oxygen levels. Increased internal oxygen could change ROS levels, and hence we further checked O_2^- and H_2O_2 accumulation. In both Desi and Kabuli, O_2^- levels were reduced in response to SNAP at 24 and 48 h but a stronger effect of SNAP was seen at 48 h (Fig. 7C). Also, Kabuli generated more H_2O_2 than Desi, which was observed as formation of a dark brown stained area (Fig. 7D). The levels of H_2O_2 were reduced in the presence of SNAP, suggesting that NO plays a role in reducing ROS. Further, we checked the expression of the alternative oxidase (AOX), which plays a role in stress tolerance via reducing ROS. Interestingly, SNAP treatment reduced AOX protein expression relative to the mock-treated control (Fig. 7E), suggesting ROS levels can determine AOX protein expression.

NO decreases lipid peroxidation and DNA fragmentation

Increased ROS can cause lipid peroxidation by oxidative degradation. Lipid peroxidation of Kabuli chickpea was significantly higher than that of Desi chickpea (Fig. 8A). NO generated from SNAP treatment led to a significant reduction in lipid peroxidation in Kabuli chickpea suggesting that a SNAP-mediated decrease in ROS (Fig. 7) can reduced lipid peroxidation (Fig. 8A). Increased ROS can cause DNA fragmentation (Gaschler and Stockwell, 2017). In this process early cleavage into high molecular mass fragments occurs followed by extensive fragmentation (Bortner *et al.*, 1995). Examination of DNA fragmentation revealed that Kabuli chickpea DNA displayed a higher fragmentation at 48 h, and interestingly SNAP caused reduced DNA fragmentation (Fig. 8B). Thus, SNAP may reduce damage to DNA most likely via reduction of ROS.

NO induces accumulation of sugars, amino acids, organic acids, and polyols but not lipids and fatty acids

In order to find out whether NO has any effect on carbohydrate, amino acid, lipid, and fatty acid metabolism, we performed metabolic analysis by GC-MS on the seeds of Desi and Kabuli treated with or without the NO donor SNAP. Sucrose levels were slightly higher in Desi in comparison with Kabuli, but in response to SNAP a 5-fold induction of sucrose was observed

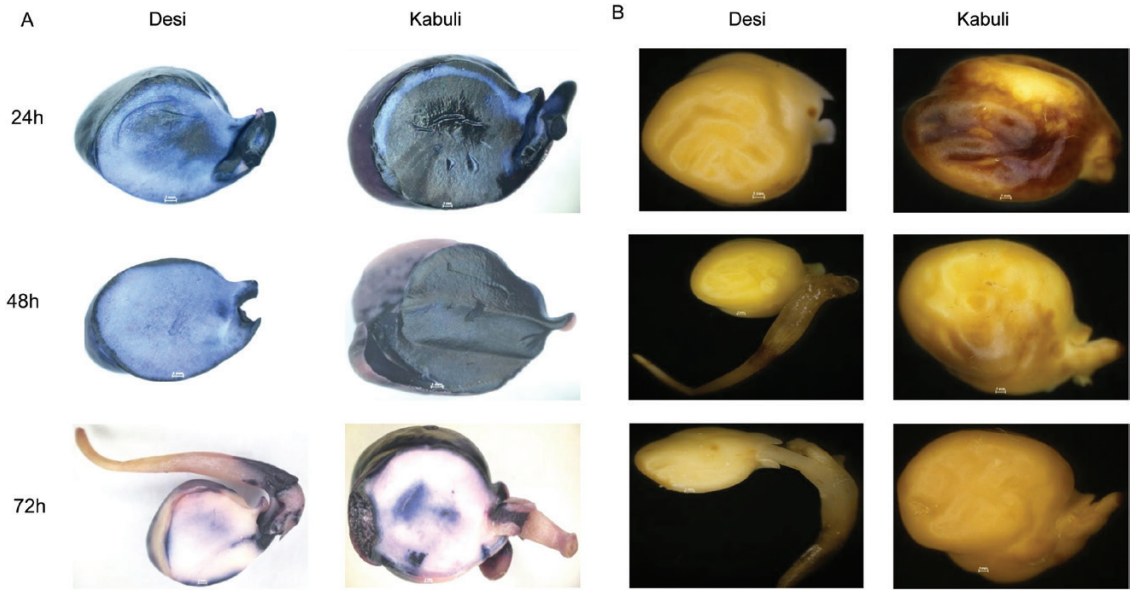


Fig. 4. ROS levels in Desi and Kabuli chickpea at different days of germination. (A) Superoxide levels determined by NBT staining. (B) Hydrogen peroxide levels determined by DAB staining. Images are representative of three independent experiments with at least six seeds.

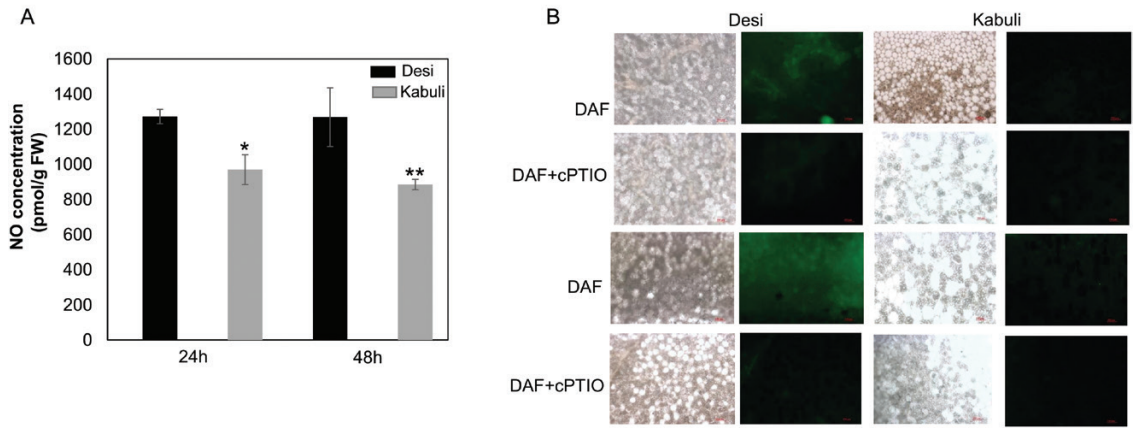


Fig. 5. NO levels in Desi and Kabuli. (A) NO levels measured by gas phase Griess reagent assay. (B) DAF-FM DA fluorescence from Desi and Kabuli after imbibition. Carboxy-PTIO control was used as indicated in figure. Image is representative of six independent seeds.

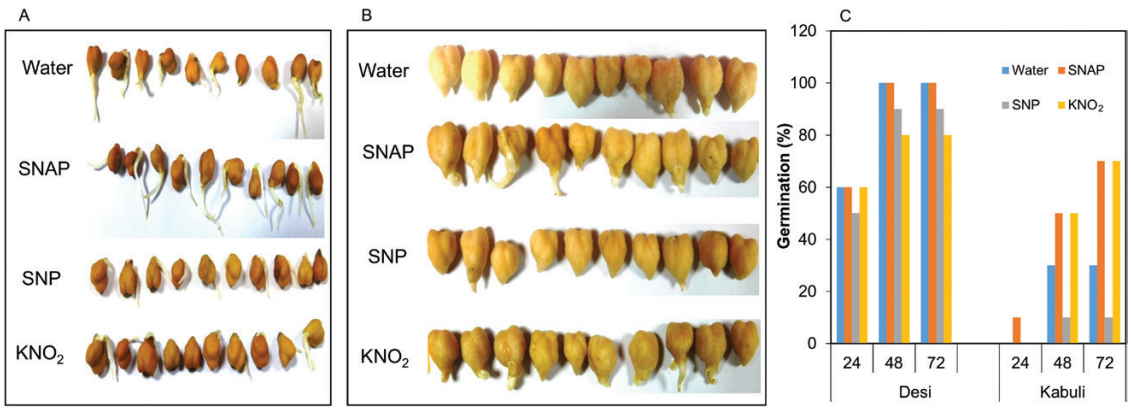


Fig. 6. Germination response of chickpea seeds in the presence of water, SNAP, SNP, and KNO_2. (A) Desi variety; (B) Kabuli variety. Image is representative of three independent experiments. (C) Germination percentage in Desi and Kabuli chickpea in response to water, SNAP, SNP, and KNO_2 treatments. Percentage is calculated from 20 seeds for each treatment. (This figure is available in colour at *JXB* online.)

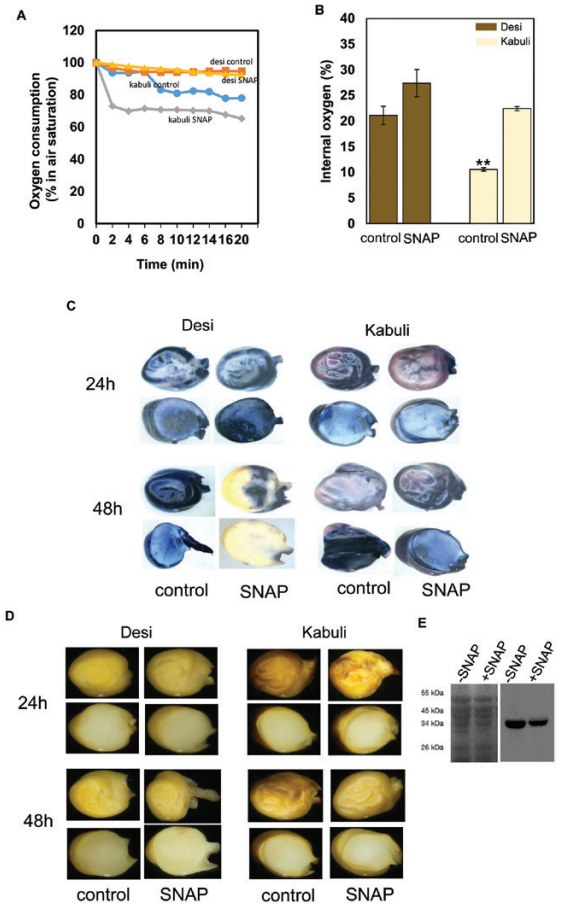


Fig. 7. (A) Respiratory oxygen consumption of Desi and Kabuli in response to SNAP treatment. Data are representative of three independent replicates. (B) Internal oxygen concentrations in Desi and Kabuli in response to SNAP treatment. Representative values are means of three replicates (n=3, ±SE). Significance was compared by t-test; $^{**}P<0.005$ considering Desi the control. (C) Superoxide levels at 24 and 48 h of SNAP treatment in Desi and Kabuli. Image is representative of 10 seeds for each time point and treatment. (D) H_2O_2 levels at 24 and 48 h of SNAP treatment in Desi and Kabuli. Image is representative of 10 seeds for each time point and treatment. (E) AOX protein levels in Kabuli chickpea in response to SNAP treatment. Blot is representative of three independent biological replicates. Left panel shows Ponceau stain displayed as loading control; right panel show AOX protein.

in Kabuli (Fig. 9A). Cellobiose levels were significantly higher in Kabuli than in Desi but the levels were not altered in response to SNAP (Fig. 9B). Galactose levels were similar in both Desi and Kabuli, but upon SNAP treatment in Desi the level was reduced. In contrast, in Kabuli significantly higher levels of galactose were observed (Fig. 9C). Methylgalactoside levels were low in both Desi and Kabuli, but upon SNAP treatment levels increased in Kabuli (Fig. 9D). *myo*-Inositol levels were low in Kabuli in comparison with Desi, but the levels increased nearly 9-fold in response to SNAP in Kabuli (Fig. 9E).

A marked increase in various amino acids was observed in response to SNAP in Kabuli. L-Glutamic acid levels were lower in Kabuli in comparison with Desi, but upon SNAP treatment the levels increased 10-fold in Kabuli in comparison with control Kabuli seeds (Fig. 9F). A similar trend was observed for L-tryptophan, where SNAP induced increased content of this amino acid in Kabuli (Fig. 9G). Phenylalanine levels were low in Desi and Kabuli, but SNAP increased content of this amino acid in Kabuli (Fig. 9H). A similar trend was observed for glycine, serine, and L-threonine, where SNAP increased these amino acid contents many fold in Kabuli (Fig. 9I–K). This response was absent in Desi. L-Valine levels were low in Kabuli in comparison with Desi, but the levels increased in response to SNAP (Fig. 9L). L-Ornithine levels were not detectable in Desi and were low in Kabuli, but SNAP slightly increased these levels (Fig. 9M). Proline levels also increased in Kabuli in response to SNAP (Fig. 9N). Examination of TCA cycle intermediates revealed that citric acid levels increased nearly 10-fold in Kabuli in response to SNAP (Fig. 10A), and malic acid levels increased nearly 3-fold (Fig. 10B). Phosphoric acid levels were low in Kabuli and levels increased in response to SNAP (Fig. 10C). Levels of polyols such as pinitol and glycerol were low in Kabuli in comparison with Desi, but in response to SNAP a marked increase was observed (Fig. 10D, E).

Despite changes in amino acids and organic acids, there were only slight changes in lipids and fatty acids. Saturated fatty acids were slightly higher in Kabuli than in Desi but in response to SNAP a significant change was observed in Desi but not in Kabuli (Fig. 10F). Monounsaturated fatty acid content slightly increased in response to SNAP in both Desi and

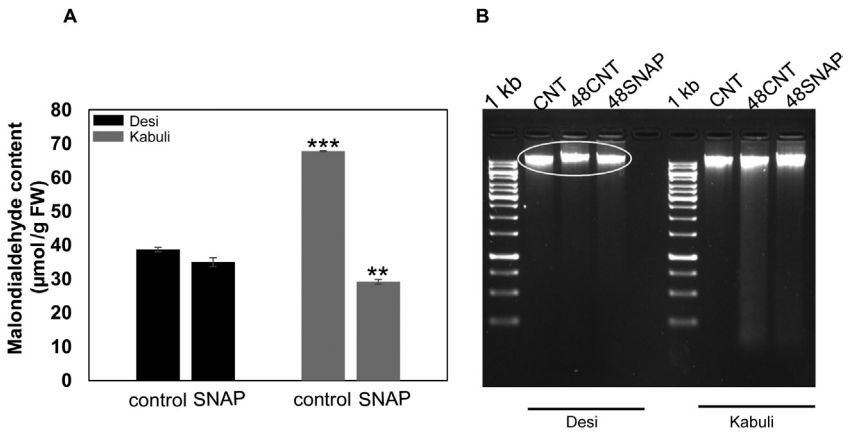


Fig. 8. (A) Malondialdehyde content of Desi and Kabuli chickpea seeds at 48 h post-imbibition during seed germination. Significance was determined by t-test; $^{**}P<0.005$, $^{***}P<0.0001$ considering Desi as control. (B) Genomic DNA fragmentation of Desi and Kabuli at 48 h in the presence or absence of SNAP. Gel image is representative of four independent experiments.

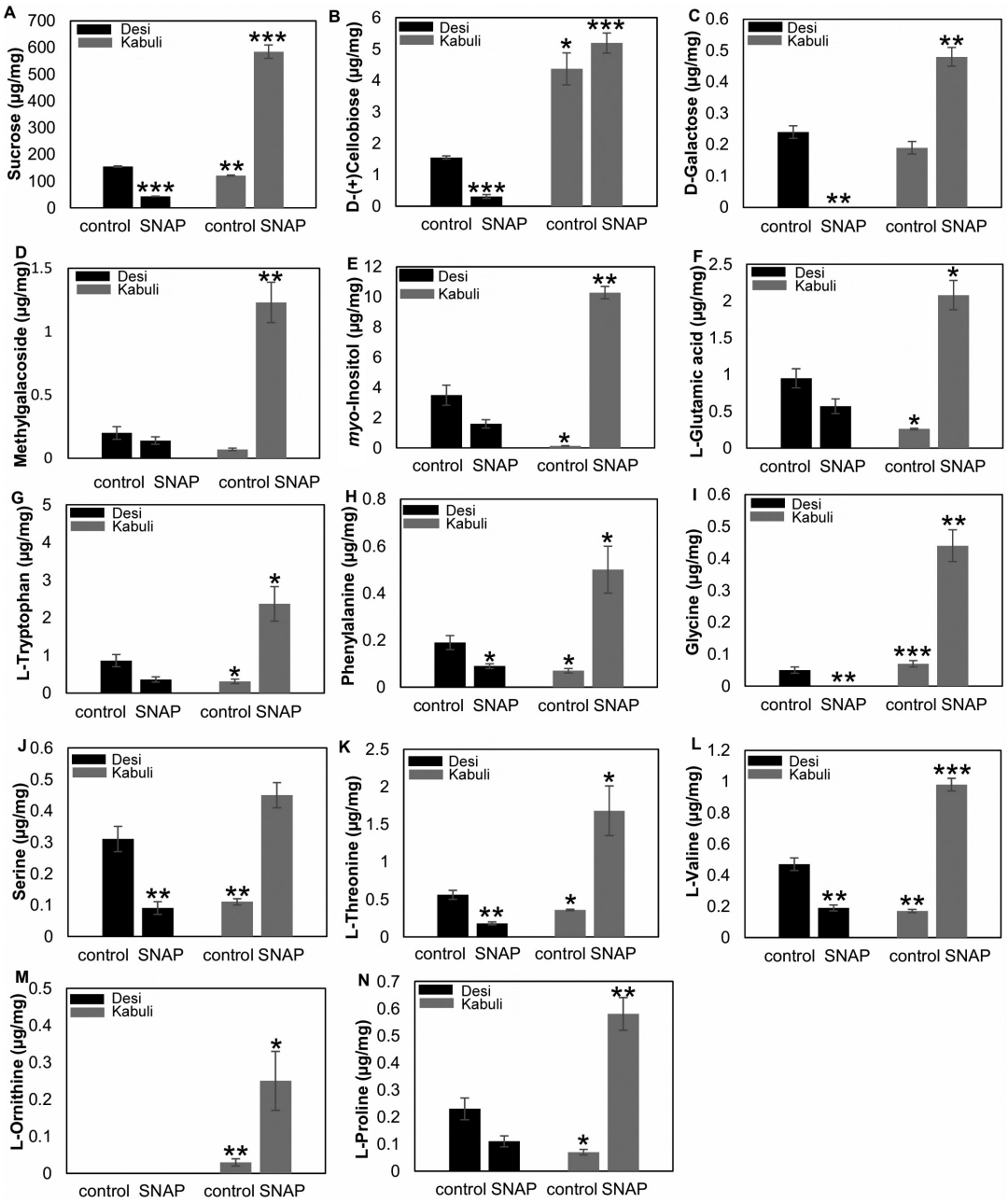


Fig. 9. Different types of carbohydrates (A–E) and amino acids (F–N) analysed in chickpea genotypes after 48 h germination. (A) Sucrose, (B) D-(+)-cellobiose, (C) D-galactose, (D) methylgalactoside, (E) *myo*-inositol, (F) L-glutamic acid, (G) L-tryptophan, (H) phenylalanine, (I) glycine, (J) serine, (K) L-threonine, (L) L-valine, (M) L-ornithine, and (N) L-proline. Values are means of three replicates (n=3, ±SE). Asterisks indicate significant between Desi and Kabuli (t-test); *P<0.05, **P<0.005, ***P<0.0001.

Kabuli (Fig. 10G). Polyunsaturated fatty acids did not change in response to SNAP (Fig. 10H). Total polyunsaturated fatty acids/saturated fatty acids did not change in Kabuli in response to SNAP whereas in Desi the ratio slightly decreased (Fig. 10I). Interestingly a slight increase in $n6/n3$ was observed in Kabuli in response to SNAP (10J). Fatty acids such as C14:1 and C15:1 significantly decreased in Desi in response to SNAP but in Kabuli there were no significant changes observed (Fig. 10K, L). C16:0 levels did not alter in response to SNAP (Fig. 10M). In response to SNAP C16:1 was decreased in Desi, whereas in Kabuli there was no response to SNAP (Fig. 10N); the opposite was observed for C 18:0 (Fig. 10O). Strikingly C18:1 levels increased in both Desi and Kabuli in response to SNAP (Fig. 10P). C18:2 levels decreased in response to SNAP (Fig. 10Q). There was no significant change observed in C18:3 (Fig. 10R). Strikingly C20:0 increased in response to SNAP in both Desi and Kabuli (Fig. 10S).

NO induces genes involved in glycolysis, sucrose metabolism, and the cell cycle

NO donor treatment may also influence various genes involved in glycolysis, sucrose metabolism, and the cell cycle during germination. Hence we checked expression of these genes in response to SNAP at 30 min and 48 h in Kabuli chickpea after SNAP treatment. The expression of *hexokinase 1* was up-regulated 4-fold after 30 min of SNAP treatment,

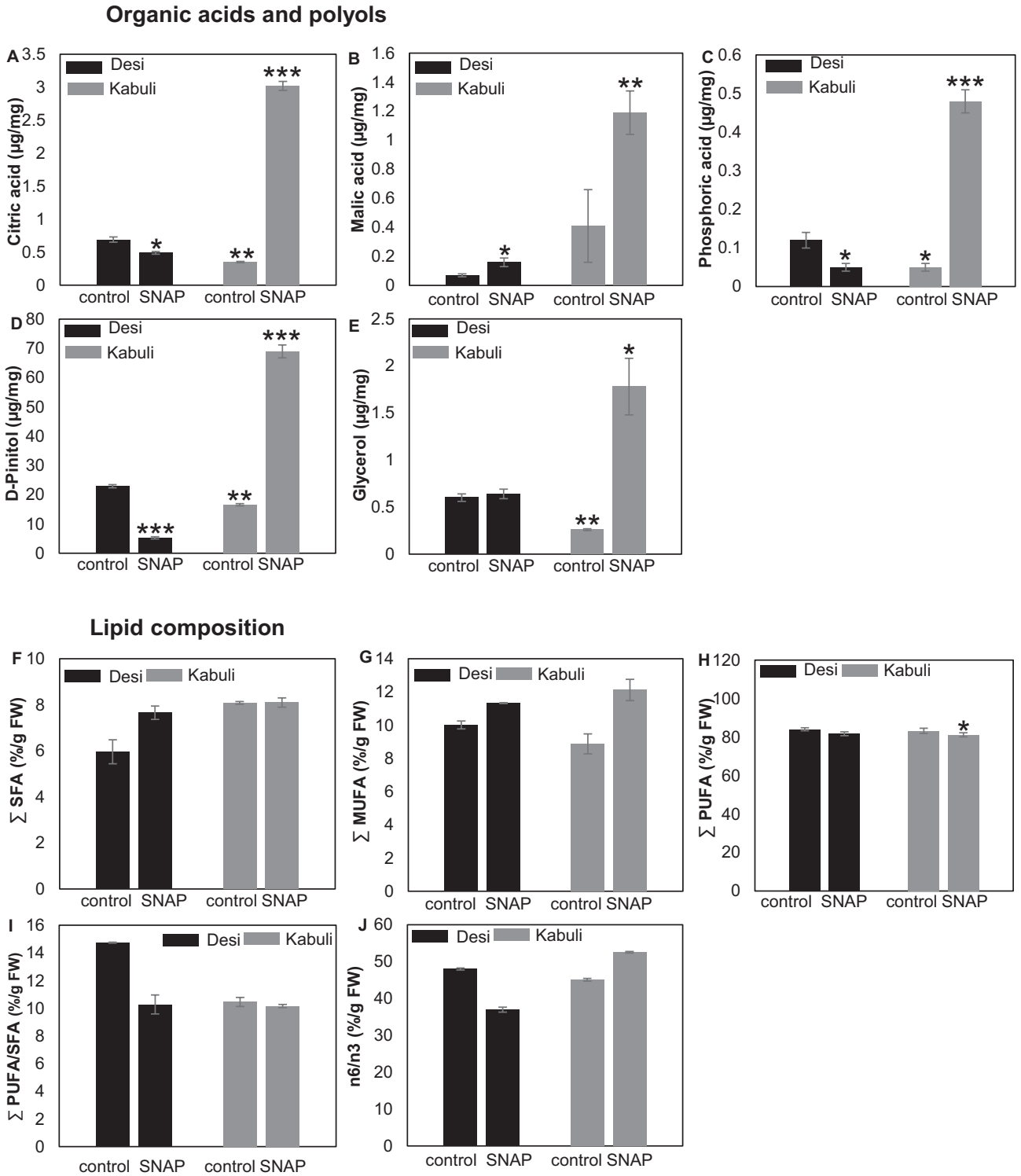


Fig. 10. (A–E) Organic acids (A–C) and polyols (D–E) of chickpea genotypes after 48 h germination post-imbibition. (A) citric acid, (B) malic acid, (C) phosphoric acid, (D) D-pinitol, and (E) glycerol. Representative values are means of three replicates (n=3, ±SE). Significance was determined by t-test; *P<0.05, **P<0.005, ***P<0.0001. (F–J) Total lipid content of chickpea genotypes after 48 h germination. (F) Saturated fatty acids (SFA), (G) monounsaturated fatty acids (MUFA), (H) polyunsaturated fatty acid (PUFA), (I) ratio of PUFA/SFA, and (J) omega-6/omega-3 essential fatty acids ($n6/n3$). Representative values are means of three replicates (n=3, ±SE). Significance was determined by t-test; *P<0.05. (K–S) Different fatty acids analysed in chickpea genotypes after 48 h germination: (K) methyl Z-11-tetradecenoate (C14:1), (L) 10-pentadecanoic acid, methyl ester (C15:1; n–5), (M) hexadecanoic acid, methyl ester (C16:0), (N) 9-hexadecenoic acid, methyl ester (C16:1; n–7), (O) octadecanoic acid, methyl ester (C18:0), (P) 9-octadecenoic acid, methyl ester (C18:1; n–9), (Q) 9,12-octadecadienoic acid, methyl ester (C18:2; n–6), (R) 9,12,15-octadecatrienoic acid, methyl ester (C18:3; n–3), and (S) eicosanoic acid, methyl ester (C20:0). Representative values are means of three replicates (n=3, ±SE). Significance was determined by t-test; *P<0.05, **P<0.005.

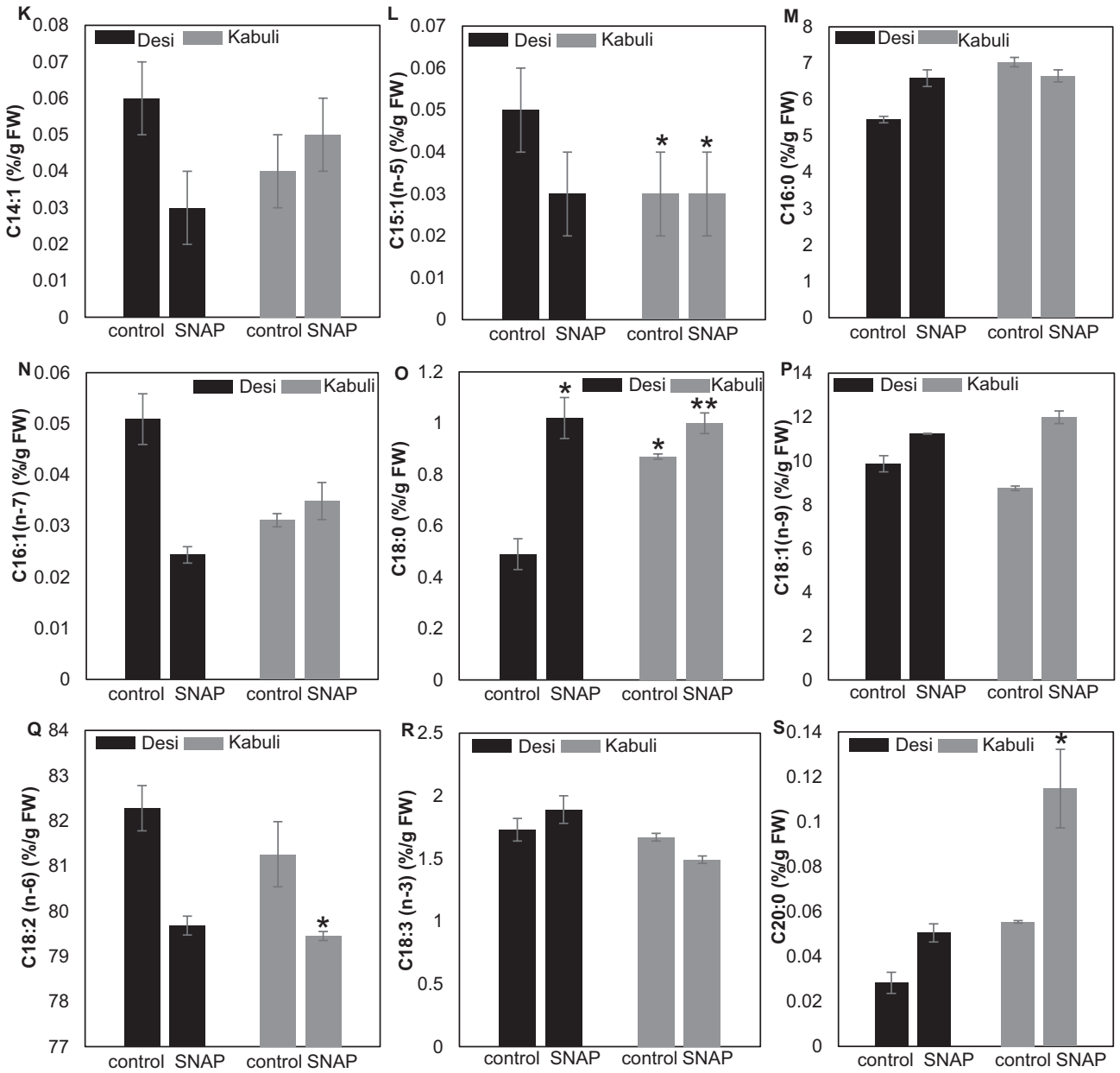


Fig. 10. Continued

whereas this gene was significantly down-regulated at 48 h post-SNAP treatment (Fig. 11A). *Phosphofructokinase 6-like* was up-regulated nearly 20-fold in response to SNAP at 30 min post-treatment, whereas this gene was down-regulated at 48 h in the mock-treated control as well as in response to SNAP (Fig. 11B). The gene encoding the final enzyme of glycolysis, pyruvate kinase, was up-regulated nearly 30-fold 30 min post-SNAP treatment, whereas at 48 h post-SNAP treatment it was up-regulated only 4-fold (Fig. 11C). The *sucrose synthase* gene was up-regulated nearly 10-fold 30 min post-SNAP treatment, whereas this gene was down-regulated at 48 h post-SNAP treatment (Fig. 11D). *Alpha amylase* gene expression increased 750-fold 30 min post-SNAP treatment, whereas this gene was down-regulated at 48 h post-SNAP treatment (Fig. 11E). The expression of the cell cycle gene *cyclin-D4-1-like* was increased 1200-fold 30 min post-SNAP treatment, whereas this gene was not induced at 48 h post-SNAP treatment (Fig. 11F). Another cell cycle gene, *cyclin-B1-4-like*, was also up-regulated 2-fold at 30 min post-SNAP treatment, whereas this gene was not induced at 48 h post-SNAP treatment (Fig. 11G).

[13C]Glucose feeding experiments revealed NO enhances glucose uptake in Kabuli and causes glucose oxidation via glycolysis and/or the pentose phosphate pathway

In order to further elucidate the role of NO in glucose oxidation during germination, we undertook parallel [^{13}C]glucose feeding experiments. One per cent (w/v) labelled glucose [either uniformly labelled 40% [$^{13}C_6$]glucose or a combination of positionally labelled [1-^{13}C]glucose + [$^{13}C_6$]glucose (in a

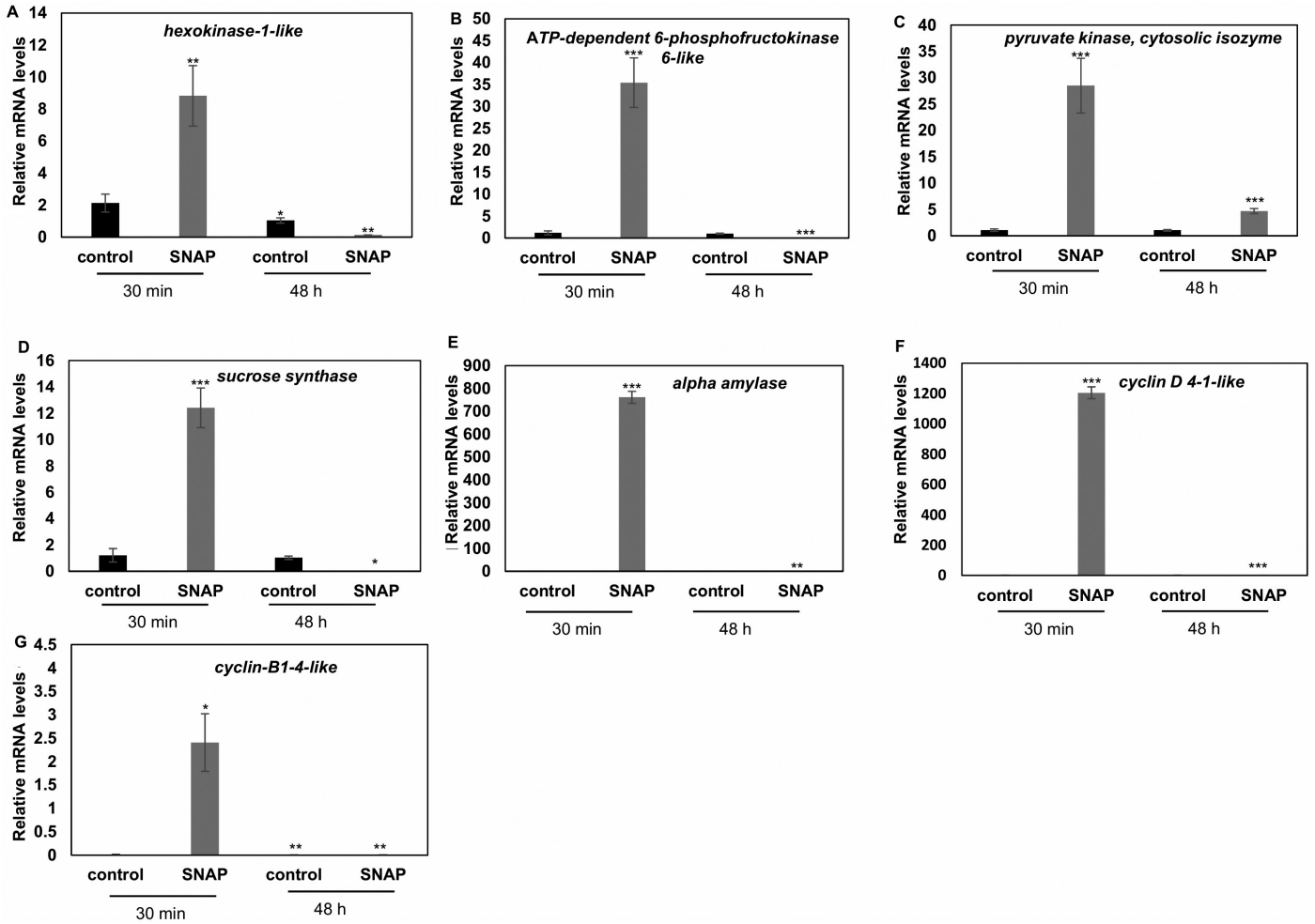
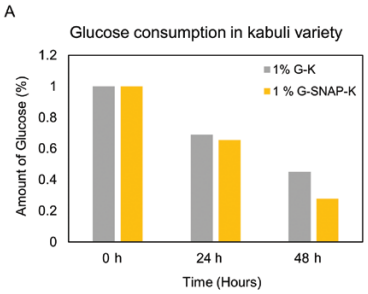


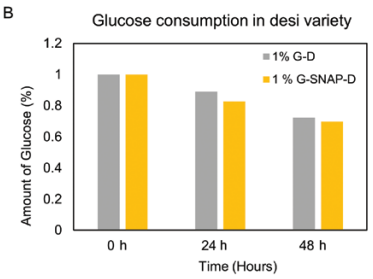
Fig. 11. Expression analysis of carbohydrate metabolism (A–E) and cell cycle (F, G) genes of Kabuli chickpea. (A) *Hexokinase-1-like*, (B) *ATP-dependent 6-phosphofructokinase 6-like*, (C) *pyruvate kinase, cytosolic isozyme*, (D) *sucrose synthase*, (E) *alpha amylase*, (F) *cyclin-D4-1-like*, and (G) *cyclin-B1-4-like*. Representative values are means of three replicates (n=3, ±SE). Significance was determined by t-test; *P<0.05, **P<0.005, ***P<0.0001.

ratio of 60:40)] was supplied exogenously to Desi and Kabuli seeds in the presence or absence of SNAP; we observed the consumption rate of glucose (using ^{1}H-NMR) as well as label incorporation into metabolites (using GC-MS). The ^{13}C incorporation into various metabolites retro-biosynthetically reports on the central metabolic pathway activities. Kabuli consumed 31% and 55% glucose at 24 and 48 h, respectively, whereas in response to SNAP the consumption was 35% and 72%, respectively, suggesting that NO generated from SNAP promotes oxidation of storage carbohydrates in order to facilitate germination. In Desi the consumption was 12% and 28% at 24 and 48 h, respectively, whereas in response to SNAP the consumption was 18% and 31% at 24 and 48 h post-SNAP treatment, respectively, suggesting a greater influence of NO on glucose consumption ability in Kabuli chickpea (Fig. 12A).

The mass isotopomer distributions of free alanine (m/z 116–118) in soluble extracts from [1-^{13}C]glucose + [$^{13}C_6$]glucose (60:40) as well as 40% [$^{13}C_6$]glucose feeding experiments confirmed the oxidation of exogenous glucose supplements in both Kabuli and Desi during germination in either the absence or the presence of SNAP (Fig. 12C). The average ^{13}C incorporated into alanine in the SNAP-treated Desi variety (16.46%) was higher than in the control (11.74%). As alanine labelling represents the labelling of the pyruvate (precursor), it can be inferred that the exogenous glucose oxidation via glycolysis and the pentose phosphate pathway was higher when treated with SNAP. However, in the case of Kabuli, there was also a slight increase in the average ^{13}C incorporation (into alanine) between control (9.72%) and SNAP-treated (10.73%) plants but this was marginal and probably due to the differential contribution of pre-existing glucose/carbohydrate reserves. The extent of labelling of free alanine in [$^{13}C_6$]glucose feeding was about 20% in both the varieties, confirming the contribution of pre-existing storage pools of alanine or oxidation of other storage carbohydrates during germination.

Given that the SNAP-treated Desi variety had a higher ^{13}C label incorporation into soluble alanine, we also investigated if this contributes to *de novo* protein synthesis. Amino acids derived from protein hydrolysates were subjected to GC-MS to measure ^{13}C incorporation. Surprisingly, amino acids (including alanine) derived from protein hydrolysates were not labelled (see Supplementary Table S2), highlighting that exogenous glucose does not contribute to *de novo* protein biosynthesis. It can be inferred that the glucose oxidation during germination at the level of glycolysis and the pentose





C

Feeding		Average ^{13}C in Alanine (%)	
		Desi	Kabuli
Unlabelled glucose (^{12}C)	Control	1.55±0.21%	1.70±0.25%
	SNAP	1.86±0.24%	2.17±0.42%
[1-^{13}C]glucose + [$^{13}C6$]glucose (60:40)	Control	11.79±0.27%	9.72±1.03%
	SNAP	16.46±3.47%	10.73±0.16%
40% [$^{13}C6$]glucose	Control	19.93±0.46%	17.27±1.04%
	SNAP	19.96±0.97%	15.55±0.68%

Fig. 12. (A, B) Amount of glucose (%) consumed by Kabuli (A) and Desi (B) seeds during germination at 24 and 48 h in presence or absence of SNAP. The amount of glucose in the germination media was quantified using ^{1}H-NMR. (C) Average ^{13}C incorporation in soluble alanine of Desi and Kabuli chickpea seeds subjected to exogenous glucose for 48 h in absence and presence of SNAP. Mass isotopomer distribution of alanine-2TMS fragment (*m/z* 116–118) measured by GC-MS. [1-^{13}C]Glucose represents ^{13}C positionally labelled at carbon C1 of glucose, while [$^{13}C_6$]glucose represents the uniformly labelled carbons, i.e. all positions C1–C6. (This figure is available in colour at *JXB* online.)

phosphate pathway might provide energy and reducing power required for the germination process.

A homozygous RIL (Desi 372 × Kabuli 1088) hybrid has increased NO levels, decreased respiration and ROS, and subsequently an increased germination rate

Since NO plays a major role in the regulation of respiration and increased internal oxygen, we employed a RIL hybrid that originated from an intercross between Desi 372 and Kabuli 1088. This hybrid had increased NO levels relative to Kabuli (Fig. 13A). Also, the respiratory rate of this hybrid was lower than Kabuli, suggesting that increased NO in the hybrid could reduce the respiratory rate (Fig. 13B). The reduced respiratory rate in Kabuli can affect internal oxygen levels, and hence we measured internal oxygen levels in the hybrid. At 2 mm depth internal oxygen levels were approximately 15% in both varieties, whereas at 4.5 mm depth the internal oxygen levels were 5% in the hybrid and only 0.8% in Kabuli (Fig. 13C), suggesting that internal oxygen levels were positively correlated with NO and negatively correlated with the respiratory rate. Internal oxygen levels can also impact on ROS. Hence, we compared O_2^- levels in Kabuli and the hybrid line. O_2^- levels were low in the hybrid (Fig. 13D) suggesting that improved internal oxygen might play a role in reducing ROS.

We also determined the potential impact of increased NO and internal oxygen on germination. Strikingly the hybrid germinated faster than Kabuli (Fig. 13E), suggesting that increased NO has a positive effect on the germination of the hybrid.

Discussion

Seed germination requires energy, to fulfil the energy demand for metabolism. Respiration increases from the dormant stage to germination to meet that energy demand. This increased respiration can deplete oxygen quickly inside the tissue, and hence fine-tuning of oxygen consumption is needed in order to avoid the tissue becoming anoxic (Zabalza *et al.*, 2009). The oxygen depletion becomes more problematic in bulky tissues where diffusion of oxygen from the atmosphere to internal tissues becomes limited. Desi and Kabuli have different seed sizes. Seeds of Desi 372 have an approximate weight of 150 mg whereas those of Kabuli 1088 are 300 mg. The Kabuli variety has bigger cotyledons and greater weight than Desi. Our experiments on germination revealed that Desi germinates more quickly (Fig. 1). Our initial respiratory experiments suggested that Kabuli respires faster than Desi. Even after 72 h (Fig. 1) Kabuli still showed very slow germination. Examination of respiration revealed that Kabuli has a higher respiratory rate than Desi at 24 h and an even higher respiratory rate at 48 h (Fig. 2). The increased respiratory rate and increased cotyledon size may be responsible for the decrease in internal oxygen levels in Kabuli.

NO is known to affect mitochondrial respiration, via reversible inhibition of the cytochrome pathway of mitochondria (Millar and Day, 1996). Previously, it was shown that this inhibition can increase oxidative phosphorylation capacity (Clerc, 2007) in a redox-sensitive manner by decreasing the slipping in the proton pumps. NO inhibits COX activity. This can cause a decrease in ATP and hence an increase in oxidative phosphorylation efficiency (Clerc, 2007). The inhibition of COX by NO also helps maintain a steady state of oxygen at the frontier of anoxia (Benamar *et al.*, 2008; Tschiersch *et al.*, 2012). This autoregulatory role of NO helps in oxygen homeostasis under hypoxia. Hence, NO levels could help fine-tune respiration to prevent the tissue become anoxic. Internal oxygen measurements at different depths of tissue revealed that Kabuli has a lower internal oxygen

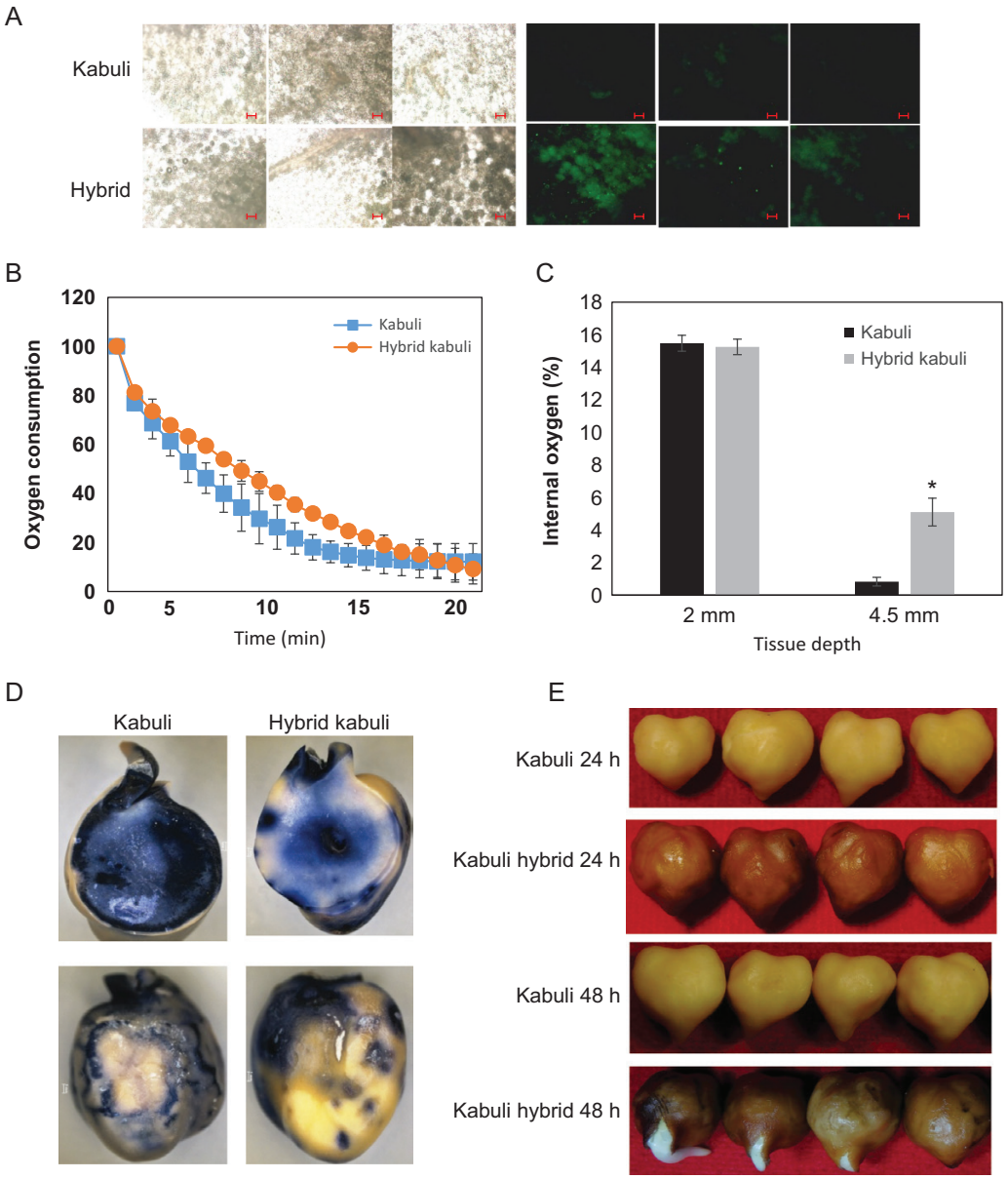


Fig. 13. (A) NO levels in Kabuli and hybrid Kabuli. Scale bars: 100 µm. (B) Respiratory oxygen consumption in Desi and hybrid Kabuli. Data are average of three independent measurements. (C) Internal oxygen percentage. Data are average of four independent replicates. Significance was determined by *t*-test; **P*<0.05 between Kabuli and hybrid Kabuli. (D) Superoxide levels of Kabuli and hybrid Kabuli. Representative of four independent replicates. (E) Germination assay in Kabuli and hybrid Kabuli. Date were from four independent replicates.

than Desi (Fig. 3). This reduced internal oxygen is probably due to fast respiratory rates and different levels of NO, and hence we checked the NO levels using DAF-FM DA and gas phase Griess reagent assays. Both measurements revealed that Kabuli has lower NO levels than Desi. This lower level of NO can relieve mitochondrial respiration, and hence Kabuli has a higher respiratory rate. This change in respiratory rate is not due to a differential capacity of the respective mitochondria for respiration. Isolated mitochondria from Desi and Kabuli showed almost similar respiratory rates (253 *vs* 259 µmol (mg protein)$^{-1}$ h^{-1}) (data not shown). The NR activity in Desi was 2.2±0.2 µmol g FW^{-1} h^{-1} whereas in Kabuli it was 0.8±0.3 mol g FW^{-1} h^{-1}, suggesting that increased NR activity is most likely responsible for increased NO production in Desi. Since Kabuli has a larger size and increased respiration this caused a drop in internal oxygen. A drop in internal oxygen can have a drastic negative effect on energy production during respiration as oxygen acts as a terminal electron acceptor in the respiratory electron transport chain. Under hypoxic conditions plant tissues generate energy via induction of fermentation and also via alternative routes, such as the non-symbiotic haemoglobin–NO cycle. The avidity for oxygen of non-symbiotic haemoglobin is extremely high (K_D ~2 nM) and this protein remains oxygenated at oxygen levels two orders of magnitude below that required for saturation of COX, and hence the maintenance of hypoxic condition is essential (Gupta and Igamberdiev, 2011)

Nitrite is a limiting factor for NO production (Planchet *et al*., 2005). Our results suggest that Kabuli has reduced levels of nitrite (see Supplementary Fig. S1). Recently it was shown

that nitrite is important for maintaining mitochondrial structure and function under hypoxia (Gupta *et al.*, 2017), and hence Kabuli may be unable to protect mitochondria under completely anoxic conditions due to reduced levels of nitrite. Previously it was shown that under anoxic conditions mitochondria produce more NO than in hypoxia (Gupta *et al.*, 2005). The K_i (oxygen) for mitochondrial nitrite-dependent NO production is 0.05%. If that is the case, Kabuli should produce more NO due to a drop in internal oxygen, but in our case we found reduced levels of NO in Kabuli in comparison with Desi (Fig. 5). This is probably due to a nitrite limitation in Kabuli (Supplementary Fig. S1). Supplementation of nitrite or treatment with the NO donor SNAP improved germination capacity (Fig. 6).

In the case of SNAP supplementation, we found several positive effects: SNAP (i) decreased respiration, (ii) improved internal oxygen, and (iii) reduced ROS.

ROS are beneficial if produced in low concentrations. If produced in high concentrations they damage proteins, DNA, RNA, and lipids, and alter various enzyme activities. Previously it was shown that hypoxia (Vergara *et al.*, 2012) induces ROS. In Kabuli we found increased ROS levels in comparison with Desi (Fig. 4). This increase is most likely due to reduced internal oxygen levels. In response to SNAP there were reduced levels of ROS. This reduction is most likely due to reduced respiration and increased internal oxygen (Fig. 7). SNAP treatment also prevented excess oxidative degradation of lipids via lipid peroxidation (Fig. 8). NO has a positive effect on induction of various antioxidants (Lu *et al.*, 2014). Since higher levels of NO can help in removal of ROS, NO can prevent DNA damage. SNAP treatment reduced DNA fragmentation in Kabuli, further supporting the role of NO in protection of DNA via reducing ROS levels.

Another positive effect of NO is the induction of cell cycle genes during development (Correa-Aragunde *et al.*, 2006). In the case of Kabuli, the supplementation of NO positively regulated cyclin B and cyclin D, suggesting that NO might even accelerate the cell cycle during germination (Fig. 11).

Examination of amino acid levels in the presence or absence of SNAP in Kabuli revealed a marked accumulation of amino acids. Previously it was shown that inhibition of aconitase by NO leads to an increase in total amino acids (Gupta *et al.*, 2012). Hence, our results can be explained via the effect of NO on aconitase. This was further supported by the observation that SNAP increased citrate levels (Fig. 10). Citrate can have a stronger effect on the induction of AOX, but in our case even the Kabuli control had higher AOX (Fig. 7). This constitutive induction of AOX is probably due to the presence of increased residual ROS, as it was shown previously that higher ROS can increase AOX transcripts and protein levels (Vanlerberghe *et al.*, 2002; Vishwakarma *et al.*, 2015). Clearly, seed germination requires protein building blocks, and hence SNAP-accumulated amino acids may help in the germination process (Fig. 9).

Our results found a requirement for NO in limiting the germination of Kabuli. The hybrid that originated from a cross between Desi has Kabuli has increased NO levels and thus increased internal oxygen and concomitantly reduced levels of ROS. Hence, a significant improvement in germination can be observed in this hybrid (Fig. 13).

Taken together our findings highlight the importance of NO in the regulation of respiration, internal oxygen and ROS homeostasis, which can become crucial factors in seed germination. Breeding programmes focused on identifying high NO producing lines in various crops can help increase germination capacity and hence improve food security.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Nitrite content in Desi, Kabuli and hybrid chickpea.

Table S1. Primer list.

Table S2. Average ^{13}C of amino acid fragments obtained from GC-MS of protein hydrolysates of Desi and Kabuli chickpea seeds in the presence and absence of SNAP for 48 h.

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