Boron Sensitivity and Storage in Brassica Napus and Its Potential for Clubroot Management

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Abstract

Clubroot [Plasmodiophora brassica] severity in canola (Brassica napus) can be suppressed by addition of boron (B), but excess B produces phytotoxicity. However, some lines of a closely related species, B. rapa, are relatively insensitive to high levels of B. Assessment of 150 accessions of B. napus and B. rapa treated with 0, 8 and 16 kg B ha-1 as disodium octaborate tetrahydrate (trade name Solubor) identified several lines that were relatively insensitive to high levels of B. In 2015, 88 lines were planted in a clubroot-infested plot in Ontario, Canada in a replicated split-plot design, where the subplot treatments were 8 kg ha-1 of B versus a non-treated control. Boron-insensitive lines generally had slightly lower clubroot severity than sensitive lines, even in the control with no added B. Application of B reduced clubroot slightly in the most sensitive lines, but produced a large and significant reduction in severity in the most insensitive lines. Assessment of five insensitive lines under controlled conditions supported the observation that application of B substantially reduced clubroot severity in insensitive lines. Comparison of two sensitive and two insensitive lines each of B. napus and B. rapa using Boron K-edge X-ray absorption near-edge structure (XANES) spectra from a synchrotron-based soft-X-ray beamline showed that some insensitive lines were able to extract B efficiently from a soil that contained very low levels of B. The spectra of sensitive and insensitive lines differed when additional B was applied, which demonstrated that the insensitive lines stored B (and other elements such as S and P) differently than the sensitive lines. Differences in the amount and type of storage likely affect the susceptibility to clubroot, and merits further study.

Background

Canola (*Brassica napus* L.) is grown in temperate regions around the world, but is especially important in Canada, where it contributes more than $26.7 billion CDN to the Canadian economy [1]. Almost all of the canola produced in North America is grown on the Canadian Prairies. Clubroot caused by *Plasmodiophora brassicae* Woronin is spreading rapidly on the Prairies [2] and represents an important threat to the industry because there is currently no effective management strategy for dealing with many of the new pathotypes that have been identified since 2013 [3].

Addition of small amounts of boron (B) can reduce clubroot severity under field conditions [4]. It delays pathogen development in root hairs and the root cortex [5, 6, 7]. A drench application at 4 kg B ha^{-1} reduced clubroot incidence and severity on canola grown at a site with very high soil organic matter (~70%), called a muck soil [7] Higher rates (8 and 16 kg B ha^{-1}) reduced clubroot even more, but also resulted in phytotoxicity and reduced seedling establishment, although most plants eventually recovered [7].

Boron is an essential micronutrient for growth and development of vascular plants. Insufficient B is the most common micronutrient deficiency in field crops [8], especially in humid regions, but the issue is also the least studied [9]. Canola has a high demand for B [10, 11] and tolerates slightly higher levels of B than many other crops [8], although the range between deficiency and toxicity is narrow. Boron-deficient

cruciferous crops exhibit stem cracking and hollowing, shifts in root growth and distribution, and prolonged flower retention. A reduction in above-ground dry weight has also been observed [12, 7, 13]. Rapeseed cultivars with some tolerance to B-deficiency have been identified [14, 15] and molecular mechanism for tolerance to low levels of B was suggested to be associated with a major QTL, *BnaA03NIP5:1b*, an ortholog of the boron influx transporter [16, 17] and the boron efflux transporter gene *BnaC04BOR1;1c* in Arabidopsis [18, 19].

Boron toxicity, although rare under field conditions in Canada, is common in other regions of the world, where it is associated with cupping and burning of leaf margins, appearing first in older leaves [12, 7]. Tolerance to high levels of boron occurs in many plant species, and the mechanism of action of tolerance hasbeen studied in a few crops. In barley, wheat and Arabidopsis, tolerance to high B-toxicity in B-insensitive lines is mediated by reduced expression of the boron influx transporter gene NIP 5:1 and boron efflux transporter gene BOR1. Reduced expression lowers the passive influx of B from the root surface into the root xylem when the concentration of B in soil is high. Also, increased expression of boron efflux transporter gene *BOR4* removes excess B from their roots, reducing B transport to the foliage [20,21, 22, 23, 24, 25]. A similar mechanism has been demonstrated in *B. rapa* [26, 27]. When soil B is high, 99% of the B taken up by B-sensitive lines accumulated in leaves, particularly in leaf margins. In contrast, B-insensitive lines retained a high proportion of B in the taproot [26, 27]. No studies on the occurrence of high B-insensitivity in *B. napus* are known. There is also a lack of information on the speciation of B linked to cell-wall changes in different tissues for any crop. Similarly, the precise location and impact of B in B-sensitive and B-insensitive lines of *B. rapa* have not been studied in detail and have not been examined at all in *B. napus*.

The mechanism underlying the effect of B on infection and symptom development caused by *P. brassicae* is not well understood. Some authors have suggested that increased B in roots may result in the production of thicker cell walls and stronger bonds in cell-wall lignin [28]. These changes would both contribute to making roots stronger and possibly less susceptible to clubroot-induced breakdown [29].

In studies of a related pathogen, *Olpidium brassicae* (Wor.) Dang, the movement of zoospores was static at 30 min following *in vitro* treatment with B (sodium tetraborate) at 50 mg mL^{-1} [30]. Root hair infection was limited by 0.3 mEq L^{-1}, with higher concentrations required for total suppression [31]. Sodium tetraborate applied to Chinese cabbage (*B. rapa*) reduced clubroot incidence and severity as rates increased up to 70 ppm [6]. The development of primary plasmodia followed the same trend. Increasing pH from 6.2 to 7.2 exacerbated this trend, but rates above 30 ppm induced phytotoxicity [32, 6].

The total concentration of boron varies across the leaf, with the highest concentrations at the leaf tip and edges [33]. Intracellular B is localized within cell walls, bound to pectin or lignin, both of which contain a high number of boron binding sites. Boron may increase plant flexibility by increasing cell wall thickness and strengthening lignin bonds [28], which might represent at least a contributing factor to its effect on clubroot. The complexes formed with B are stable in water and alkaline solutions, forming gels if exposed to acidic conditions [34].

Previous studies have shown that the concentration of B extracted from plants treated with small amounts of B (e.g., 4 kg ha^{-1}) in canola root tissues are in the ng range [35] which is near the B detection limit of soft X-ray beamlines [36]. The B rates in the current study are higher, so assessment with soft X-ray was selected for the evaluations. Synchrotron-based X-ray, with high intensity and wavelength flexibility, has unique advantages relative to that produced by other X-ray machines [37, 38]. The synchrotron-based soft X-ray Variable Line Spacing Plane Grating Monochromator (VLS-PGM) beamline at the Canadian Light Source facility was used for B identification and speciation in bulk samples using Boron K-edge XANES analysis. XANES (X-ray absorption near edge structure) is an element-specific technique that tracks the local electronic and chemical structure in a sample. An edge results when a core electron absorbs energy equal to or greater than its binding energy. Edges are labeled according to the shell the core electron originates from.

The excitation energy presented in the XANES spectra was chosen to cover the boron K-edge (185 to 210 eV) The K-edge for element B is 188 eV, which is equal to than the binding energies of core electrons. At the input energy edge, the 1s core electrons are excited and transition to previously unoccupied 2p orbitals. Such transitions follow the dipole selection rules and give rise to a so-called absorption edge jump because of the vertical spike that occurs in the XANES spectra.

The objectives of the present study were to identify accessions of *B. napus* and *B. rapa* that were relatively insensitive to B, validate that application of B reduced clubroot severity in these B-insensitive accessions, and examine how, where and what form or species of B was stored in B-insensitive and sensitive plants of *B. napus* and *B. rapa* using the B-K edge XANES available from a soft X-ray synchrotron beamline. The main hypothesis was that application of optimal amounts of B to B-insensitive genotypes would result in increased uptake and storage of B in roots and other tissues, resulting in reduced development of clubroot symptoms in roots. A second hypothesis was that B was stored in different locations and different compounds in B- sensitive and B-insensitive lines of the same species.

Results

Screening accessions / Field validation

In the initial controlled environment assessment, the vast majority of lines exhibited extensive chlorosis and even defoliation (data not shown). Many of the accessions eventually recovered from the initial phytotoxicity, but several accessions of *B. napus* and *B. rapa* were identified that were relatively insensitive to application of B.

In the field trial, several of the accessions that had been rated as relatively insensitive in the controlled environment study were also relatively insensitive under field conditions. There were no difference in phytotoxicity (very low) or clubroot severity (high) among accessions in the non-treated control, but there

were differences on both aspects among accessions in plots treated with 8 kg ha^{-1} B (B x accession, $P < 0.0001$, data not shown).

To examine the effect of B application on clubroot in more detail, the 9 accessions that were most sensitive to B and the 10 accessions that were least sensitive to B, based on assessment of seedling phytotoxicity, were selected for further analysis. Within this subset of accessions, the B-insensitive accessions with no added B had lower ($P < 0.0001$) clubroot severity than the B-sensitive accessions and application of B resulted in a further reduction in severity in the insensitive lines, but no reduction in clubroot on the sensitive accessions as a group (Fig. 1 and Additional file 2). However, there were no differences between the sensitive and insensitive subsets in fresh or dry weight of foliage.

Reassessment under controlled environment

Five of the most B-insensitive accessions identified in the screening studies were re-assessed in a growth room study. Seedling phytotoxicity was lower in four of the accessions and clubroot severity was lower in three accessions, R_3420, UC77 1251 and Nevin, relative to a control (Table 1). Two lines each of *B. napus* and *B. rapa* were confirmed as being relatively insensitive to B at 8 kg ha^{-1}.

B x Ca

In the examination of the interaction of B x Ca, application of B reduced clubroot severity on the B-insensitive accessions, independent of Ca. Application of Ca at 1000 kg ha^{-1}, however, slightly reduced the phytotoxicity of B on the B-insensitive accessions (Fig. 2), but did not affect clubroot severity or plant growth. Therefore, this line of study was not pursued further.

Boron K-edge XANES analysis

The B K-edge XANES spectra in roots of the four *B. napus* lines were examined. There were clear differences among the spectra of the samples associated with B application rate (0, 8, and 16 kg B ha^{-1}) and between insensitive and sensitive accessions (Figs. 3, 4). Samples with no added B exhibited clear resonance, which demonstrated that all of the roots contained B. Also, the analysis demonstrated that the insensitive accessions (Mytnickij, Prota) absorbed, accumulated and stored B in roots differently than the sensitive lines (Westar, ACS N39) (Fig. 3). There were also differences in the L-edge XANES spectra between these groups for P and S elements (Additional file 3).

In the absence of added B, roots of the sensitive lines had an edge jump (peak) at around 194eV (Fig. 3). Based on previous descriptions of the maximum energy positions, the peak at 194 eV was likely from trigonal coordinated B (free boric acid, $B(OH)_3$), which is the available form of B in plants and so the expected peak for unincorporated trigonal coordinated B-oxygen in plant species [39, 40]. This peak

demonstrated that root tissues of B-sensitive accessions mainly contained trigonal coordinated B. In contrast, the main edge jump for insensitive lines occurred at 192.5 eV (Fig. 3). The peak at 192.5 eV in insensitive lines without B treatment was most likely from B-N and B-C bonds [19, 41-43], which indicated that the insensitive lines had incorporated the B into tissues. The observation that insensitive lines incorporated more B directly into root tissues than sensitive lines may be a factor in the reduced susceptibility of insensitive lines to the development of clubroot symptoms.

When additional B was provided in soil, the spectra of root tissues were greatly changed. The main peak at 194 eV in the sensitive lines was enhanced with addition of B at 16 kg ha^{-1} compared to B at 0 kg ha^{-1} (Fig. 3c, d). Similarly, the main peak of the insensitive lines shifted from 192.5 eV to 194 eV with addition of B from 8 kg/ha to 16 kg/ha (Fig. 3a, b). The peak intensity of the main edge jump at 194 eV increased with increased concentration of B in both sensitive and insensitive lines, followed by a broad bump centered at 198 eV that is the expected peak for tetrahedral coordination B at 16 kg ha^{-1} (Fig. 3), compared with those at 0 and 8 kg ha^{-1} B treatments. The higher peak intensity indicated a large increase in the trigonal coordinated B. The large increase of trigonal coordinated B-O with a well defined peak at 194 eV following B application demonstrated that large amounts of B were being absorbed and stored in the roots. This indicated that B-insensitive lines have a higher capacity to utilize B at high B concentration.

Boron accumulation was also examined in leaves in B-sensitive and insensitive lines of *B. napus* (Fig. 4). Insensitive lines were generally better than the sensitive lines at extracting B from growth media that contained low levels of B. As with roots, sensitive lines absorbed and stored high levels of B in leaves at 8 kg ha^{-1}, but the spectra were almost unchanged at 16 kg ha^{-1} (Fig. 4c, d). The insensitive accession Mytnickij showed the strongest edge jump in leaves from the peak 192.5 eV to 194 eV, which indicated a large increase in B content (Fig. 4a). As in the roots, the peak intensity at 194 eV often increased with higher amounts of B added. However, the peak intensity at 194 eV was already dominant in leaves of insensitive accessions at 8 kg ha^{-1}, while a similarly strong response was not observed in the roots of these accessions until 16 kg ha^{-1}. This indicated that, even in relatively insensitive accessions, more B was absorbed and stored in leaves than in roots.

Boron K-edge XANES were also performed on roots of sensitive and insensitive accessions of *B. rapa* (Fig. 5). As with *B. napus,* samples with no added B exhibited clear resonance, mainly at 192.5 eV and 194 eV, which demonstrated that the roots contained B. The roots of the sensitive accessions (SRS 3399, 3406) were B-O dominant, while the insensitive accessions (SRS 3361, 3364) were B-N and B-C dominant. Added B greatly changed the B environment for roots; the peak at 194 eV was again enhanced, which indicated that there were more B-O bonds in the roots, such that the local environment became B-O dominant in both sensitive and insensitive accessions. In leaves of plants with no added B, the spectra for all of the lines were similar (Fig.5), indicating a B-O dominant environment. When B was added, the peak for B-O bonds was greatly increased, which demonstrated that B accumulated as B-O bonds in all accessions, and B was not being incorporated into tissues as B-C or B-N bonds. Also, the main peaks in P

and S-L-edge XANES spectra were stronger in the sensitive lines relative to the insensitive lines, which indicated that more P and S were stored in the sensitive lines Additional file 3).

Rhizosphere soil was also examined before and after B addition. Boron was present in the soil in the form of trigonal coordinated B with a well defined main peak at 194 eV both before and after addition of B. There was no detectable resonance in the L edge spectra of sulphur (S) and phosphorus (P), which demonstrated that the concentration of P and S in the soil was very low (Additional file 4).

Discussion

In the current study, seedlings of *B. napus* and *B. rapa* accessions were evaluated for sensitivity to high levels of boron (B), based on phytotoxicity in two growth-room studies and a field trial. Two accessions each of *B. napus* and *B. rapa* with reduced sensitivity to high B were identified and selected for further study. Previous reports had identified and studied lines of *B. rapa* with reduced sensitivity to high levels of B [26, 27], but to our knowledge this is the first study of insensitivity to high levels of B in *B. napus*. It is also the first time that a synchrotron-based XANES K-Edge analysis of boron speciation using a soft-X-ray beamline has been applied to either plant species. Finally, this is the first study of the potential for application of insensitivity to high B to suppress a disease in any host.

The field trial and controlled environment studies of B × clubroot interaction confirmed that clubroot severity could be reduced by application of B, and demonstrated that the reduction could be substantially larger in B-insensitive accessions relative to B-sensitive accessions when additional B can be applied. The initial assessment of ~150 accessions of *B. napus* and *B. rapa* to low levels of B applied under controlled conditions, and the repeated trial on 88 of the accessions (with adequate seed supplies) under heavily infested field conditions showed similar trend in B sensitivity and clubroot reaction. When the most B-sensitive accessions were compared with the most insensitive accessions, the two subsets did not differ substantially in clubroot severity in the control treatment (no added B). However, application of 8 kg B ha^{-1} reduced clubroot more in the insensitive accessions than in the sensitive accessions. This result indicated that added B helped B-insensitive accessions more than sensitive accessions in reducing susceptibility to clubroot.

Sufficient seed for subsequent testing was not available for most of the accessions, so a growth room study to confirm the interaction of B × clubroot assessed only five B-insensitive accessions against one sensitive control. Seedling phytotoxicity from added B was substantially reduced in four of the five insensitive accessions, and added B generally reduced clubroot severity relative to the non-treated control, as well as to most of the treatments applied to the B-sensitive accession. Based on these studies, two B-insensitive accessions of *B. napus* (Mytnickij, Prota) and *B. rapa* (SRS 3361, SRS 3364) were selected for further assessment.

Previous reports on reduction in clubroot severity after application of B indicated that the treatment reduced colonization of the root cortex by *P. brassicae* [32], possibly through changes in cell wall

composition or even direct toxicity to the pathogen. The effect of increased B in roots may involve the production of thicker cell walls [28] and stronger bonds in cell-wall lignin. Adding high levels of B may also affect cytokinin and auxin metabolism, both of which are altered substantially as a result of secondary infection and colonization by *P. brassicae* [44]. These changes could all contribute to making the plant less susceptible to clubroot-induced breakdown [29].

Another element that has been shown to influence clubroot severity is calcium [reviewed in 45]. A small study (replicated but not repeated) demonstrated that the interaction between B and Ca had little or no effect on clubroot severity. Application of Ca slightly reduced the phytotoxicity of B on the B-insensitive accessions, but did not affect clubroot severity or plant growth.

Novel synchrotron-based approaches are currently being used to investigate many complex problems involving plants and soil, but the potential usefulness of such approaches to assess B distribution and speciation in plants had not been investigated previously. Analysis of B-sensitive and -insensitive lines of *B. napus* and *B. rapa* using Boron K-Edge XANES analysis demonstrated that insensitive accessions stored B (as well as S and P) differently than sensitive accessions. In both species, the spectra of insensitive lines differed substantially from those of sensitive lines, with a well defined peak at 192 eV at lower B concentration. There was also a smaller peak at 194 eV in the insensitive accessions relative to the sensitive accessions, which indicated that insensitive accessions incorporated more B into B complexes in both root and leaf tissue (more B-C and B-N bonds relative to trigonal $B(OH)_3$) at low concentration. Also, insensitive accessions accumulated much more B in roots and leaves in the high added B treatment than B-sensitive accessions, without developing symptoms of phytotoxicity. In sensitive accessions, B absorption was saturated at 8 k ha^{-1}.

Previous studies indicated that the boron speciation within plant tissues observed in the current study likely consisted of unchanged boric acid, B complex binding with soluble phenypropanoids in the cytoplasm (B associated with carbonate) for detoxification of excess B, and B complex binding with insoluble carbohydrate polymers such as pectin on primary cell wall (tetrahedral $B(OH)_4$) and sucrose in phloem [46, 47, 41]. The predominant peak at 194 eV in sensitive accessions without added B may indicate the presence of free boric acid (trigonal $B(OH)_3$), which would lower pH in the cytoplasm. The predominant peak at 192.5 eV that was observed in insensitive lines grown in soil with high levels of B may indicate the presence of B binding of phenylpropanoids with B-carbonate, associated with higher lignin or phenolic acid content. More lignin and phenolic acid may contribute to reduced susceptibility to clubroot. At lower levels of added B, the peak shifted from 192.5 eV to 194eV in insensitive lines. This shift indicated that cleavage of the B-phenolics and formation of free boric acid for translocation to leaves was occurring, likely to reduce B toxicity in root tissues. This cleavage would release phonic acid, which could also contribute to increased disease resistance. At higher levels of added B, much of the B was tetrahedrally coordinated with a well-defined peak at 198 eV.

The exact form of tetrahedral-coordinated B is not known, but B has been shown to cross link with rhamnogalacturonan-II (RG-II) pectin (tetrahedral $B(OH)_4$ species) subunits to stabilize plant cell walls

[41]. At higher B concentration, insensitive lines likely cross-linked B with pectin, which would reduce B toxicity and might also contribute to reduced clubroot susceptibility. The predominant form of B in tissues of *B. napus* and *B. rapa* was trigonal $B(OH)_3$ rather than tetrahedral $B(OH)_4$. This demonstrated that the majority of B was localized in the cytosol rather than in cell walls. This supports a previous report that the concentration of B was much lower in the cell walls of *B. rapa* than in the cell sap of leaves [26].

The observation that both *B. napus* and *B. rapa* exhibit a similar pattern of response to B indicated that B-insensitivity may be common in *Brassica* spp. and so might be a factor that affects the susceptibility to clubroot. In contrast, P and S storage in roots was higher for sensitive lines than for insensitive lines, but these traits do not seem to affect clubroot severity.

It is possible that high levels of B could be applied to clubroot-infested areas of field and then the infested patch seeded to a highly B-insensitive cultivar of *B. napus* to maintain canola production across a field where only a portion of the field is infested with clubroot. This approach should be equally effective against any pathotype of *P. brassicae*, which would be useful because resistance to many of the pathotypes present on the Canadian Prairies is currently not available [3]. This approach merits additional study. However, high levels of B can produce phytotoxicity in barley [48, 49], wheat [49, 50], and field pea [51, 52]. These crops are grown routinely in rotation with canola on the Canadian Prairies, so care would be needed to ensure that treatments do not adversely affect subsequent crops.

Conclusions

In the current study, two accessions each of *B. napus* and *B. rapa* with reduced sensitivity to relatively high levels of B in soil were identified. Insensitivity to high levels of B had not previously been confirmed in *B. napus*. The study confirmed that adding B to soil reduced susceptibility to clubroot in both host species, but then extended that knowledge by showing that the effect of added by was much stronger in B-insensitive accessions than in B-sensitive accessions. Synchrotron-based XANES K-edge analysis of boron speciation, used for the first time to assess B-storage in either host species, demonstrate that the B-insensitive accessions stored B differently than sensitive accessions, primarily by incorporating more B into root tissues. The resulting changes in the cell wall structure from increased incorporation of B into root tissues represents a likely mechanism to account for the reduced susceptibility to clubroot in the B-insensitive lines. This study represents the first assessment of the potential for application of insensitivity to high B to suppress a disease in any host.

Materials And Methods

Screening accessions / Field validation

Assessment of 150 accessions of *B. napus* and *B. rapa*, acquired from Plant Gene Resource Canada (Agriculture and Agri-Food Canada, Saskatoon, SK), was conducted in a growth room set to 25° / 20° C day night cycle, 16 hr photoperiod and 65% R.H. Tall narrow plastic pots (Conetainers, Stuewe and Sons,

Tangent, OR) were filled with LA4 soil-less mix and planted with a single, newly germinated seedling, in a completely randomized design with two replicates and10 plants per experimental unit. No fertilizer was added. The treatments were 0, 8 or 16 kg ha^{-1} of B (Solubor, 21% B, disodium octaborate tetrahydrate http://www.borax.com/product/solubor.aspx) applied at 21 days after planting (5 mL of a solution with 26 g B L^{-1} = equivalent to 8 kg B ha^{-1}, 10 mL = 16 kg B). The seedlings were maintained in a growth room set at at 25 / 20 C day night cycle, 16 hr photoperiod and 65% R.H.

Lines were visually assessed for symptoms of B-induced phytotoxicity, including chlorosis of leaves and defoliation, at 7 days after B application. Toxicity was determined on a scale of 0–3, where 0 = seedlings with no visual symptoms, 1 = light marginal burning of leaves, 2 = marginal burning and cupping (Additional File 1), and 3 = severe marginal burning and cupping. Toxicity for each cultivar was calculated using the equation below.

$$\text{Boron Toxicity} = \frac{\sum [(\text{class no.})\,(\text{no. of plants in each class})]}{(\text{total no. plants per sample})\,(\text{no. classes} - 1)} \times 100$$

A field trial was conducted to validate the impact of B on phytotoxicity symptoms and to assess the clubroot reaction of *B. napus* and *B. rapa* accessions on a high organic-matter soil (pH 6.0, 66% organic matter) infested with pathotype 6 of *P. brassicae* at the Muck Crops Research Station (MCRS), Holland Marsh, Ontario. On 11–13 May, 2015, 150 accessions were seeded in germination trays at a rate of 120 seeds per accession and placed in a greenhouse for establishment. The mean daily temperature ranged from 18°–25° C with 14.5 hr photoperiod and 70% R.H. Seed germination and establishment differed substantially among the accessions. After 3 wk, only 88 of the original 150 accessions had sufficient seedling establishment for inclusion in the study. These accessions were hand transplanted into the field on 8–10 June.

The study was arranged in a split-plot design with B treatment (0 vs. 8 kg ha^{-1}) as the whole plot treatments and accession as the subplot treatment, with four replicates and 10 plants per subplot. Boron was applied as a drench on June 12 using a CO_2 backpack sprayer at a rate of 1500 L ha^{-1} and concentration of 26 g L^{-1}. Heavy rain on 13–14 June likely leeched B from the root zone. No seedling phytotoxicity was observed on 19 June, so a second application of B at 8 kg ha^{-1} was made on 20 June. Boron toxicity was assessed on 26 June as described previously and a phytotoxicity index was calculated for each accession.

The roots of each plant were visually assessed for clubbing between 13–16 July on a 0–3 scale where 0 = no clubbing, 1 ≤ 1/3 of tap root with clubs, 2 = 1/3 to 2/3, and 3 > 2/3 of the tap root clubbed. A Disease Severity Index (DSI) [53]:

$$DSI = \frac{[(\text{class no.})(\text{no. of plants in each class})]}{[(\text{total no. plants per sample})(\text{no. classes} - 1)]} \times 100$$

The above-ground biomass of 10 plants from each plot of a subset of accessions (the 10 most B-sensitive accessions and 10 most B-insensitive accessions) were collected and fresh and dry weights of above-ground biomass were assessed.

Controlled environment

A study was conducted to re-assess the 10 most B-sensitive and 10 most B-insensitive lines from the field trial under controlled conditions. The experimental design was a factorial with B application (0 or 8 kg B ha^{-1}) as one factor and accession as the second factor, in a randomized complete block arrangement with four replicates and 10 seedlings per experimental unit. Seed was germinated in germ pouches in a growth room to maximize the number of seedlings produced, but the germination of the accessions was very low and variable. There were sufficient numbers of seedlings for only two replicates of five B-insensitive accessions; Nevin, R_3420, Ghobi, GS_867, UC77_1251, GS_869 and one B-sensitive breeding line, ACS N39. No additional seed could be obtained for the other accessions, so they could not be included in the study. The study was conducted in a growth room set at 25° / 20° C day night cycle with 16 hr photoperiod and 65% R.H.

Seedlings were transplanted after 5 days and watered daily for 3 days, left dry for 2 days and treated with the equivalent of 8 kg ha^{-1} B. Each seedling was inoculated with *P. brassicae* at 5 days after boron application (10 days after transplanting) with 5 mL of a suspension containing 3.4 x 10^6 spores mL^{-1}. Clubroot severity and top weight (fresh and dry weight) were assessed as described previously.

B x Ca

A growth room study was conducted to assess the effect of levels of Ca on the phytotoxicity of B and on clubroot development. The experiment was a factorial of accession x B (0, 8, 16 kg B ha^{-1}) x Ca (0, 250, 500, 1000 kg Ca ha^{-1}), in an randomized complete block design with three replicates and 10 plants per experimental unit. The accessions were two B-insensitive accessions of *B. napus* (Haiquotian and Mytnickij), one of *B. rapa* (SRS 3364), and one B-sensitive breeding line of *B. napus* (ACS N39) as a clubroot-susceptible and B-sensitive control. The Ca (calcium carbonate, $CaCO_3$, Sigma Aldrich) was mixed into the soil-less mix prior to transplanting. Maintenance of the seedlings, application of B, and inoculation with *P. brassicae* were as described previously, except that the spore concentration was 5.0 × 10^6 spores mL^{-1}. Clubroot severity and fresh weights were assessed as described previously.

Boron K-edge XANES analysis

A study was conducted to examine the uptake and storage of B in two lines each of B-sensitive and B-insensitive *B. napus* and *B. rapa*. The study was laid out in a factorial randomized complete block design with three replicates and three plants per experimental unit. One factor was lines of *B. napus* (Westar, ACS N39 – B-sensitive; Prota, Mytnickij – B-insensitive) and *B. rapa* (SRS 3399 & SRS 3406 – B-sensitive; SRS 3361 & SRS 3364 – B-insensitive) and the second factor was levels of B applied at 0, 8 and 16 kg B ha^{-1} as Solubor. Seed was germinated for 2 days in moist paper towel, transplanted into acid-washed sand, and grown for 12 days in a growth room set at 25 / 20 C day / night cycle, 16 hr photoperiod and 65% RH. The roots and leaves were collected separately, washed, freeze-dried and ground with a mortar and pestle in preparation for Boron K-edge XANES analysis. About 5 mg of dry ground samples were collected for analysis.

There were issues with the reliability of some of the initial assessments of the *B. napus* lines for Boron K-edge XANES analysis, so additional samples were prepared as previously described, except that seedlings were transplanted to 24 cm x 32 cm plastic flats of Sunshine #3 soil-less mix (Sun Gro Horticulture, Vilna, AB).The leaf and root samples were frozen in liquid nitrogen, ground with a mortar and pestle, then freeze dried prior to analysis.

Boron K-edge, phosphorous and sulfur L-edges XANES were performed at the Variable Line Spacing Plane Grating Monochromator (VLS-PGM) beamline at the Canadian Light Source, University of Saskatchewan [54]. A high flux beamline with a recently installed Elliptical Polarizing Undulatoron (a 3rd generation synchrotron source) facilitated good detection. The VLS-PGM beamline has good flux and energy resolution required for this study at the B K-edge (1s to 2p transitions).

Each dry, powdered sample was applied onto a 0.5 cm x 0.5 cm piece of double-sided carbon tape stuck to the bottom of a glass Petri dish. Any loose powder was blown away using compressed air to form a thin layer on sample plate, and the samples were transferred into a high vacuum sample chamber, and the chamber was pumped to 10^{-7} Torr (1 Torr = 133.3 pa). The XANES spectra were collected simultaneously in two different modes: total electron yield (TEY) mode by quantifying the drain current from each sample upon exposure to X-rays, and fluorescent yield (FLY) using a microchannel plate detector [55] facing the sample at an angle of 45^{o}. The beamline slits were 100 µm x 100 µm with a resolution $E/\Delta E > 10000$. The pressure in the experimental chamber was maintained at < 1 x 10^{-7} torr for all measurements. FLY data were normalized by the I_0 current in proportion to the incident beam intensity, which was measured by placing a piece of nickel mesh in front of the sample. The energy of the beamline was calibrated against the exciton peak of BN at 192 eV. Origin 9.0 (OriginLab) was used to process the XANES data and produce the figures. Repeated scans on at least two spots of each sample were collected at room temperature. The flat pre-edge of spectra were obtained by subtracting straight line (linear baseline correction) between 187 eV and 191 eV. For better comparison of samples, an edge

step of 1 from 190 eV to 210 eV of spectra was realized by further normalization. As the electronic conductivity of plant samples were poor, the TEY spectra were noisy, so only FLY data are presented.

Statistical analysis

The Glimmix procedure in SAS 9.3 was used to analyse variance within an experiment and Tukey's test conducted to assess all pairwise comparisons between treatments and among treatment groups. Uneven field distribution of clubroot resting spores resulted in clusters of plots with very low clubroot severity results. These artifacts, along with several outliers identified using Lund's test, were removed from the data. Statistical analyses were carried out at $\alpha = 0.05$.

Declarations

•Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author's contributions

BDG, AM and MRM conceived the study, AM, DW, RL and JYT conducted the assessments and analysis, BDG, MRM, DW, CK and GP contributed to data interpretation, and BDG developed the manuscript. All authors have reviewed the ms and support the conclusions.

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Figures

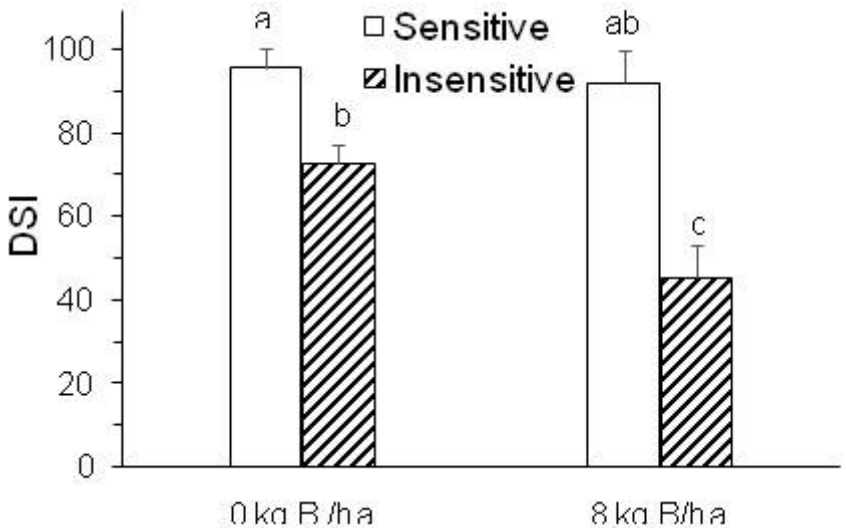

Figure 1

Effect of a drench application of boron at 8 kg ha-1 on clubroot severity (disease severity index, DSI) of the 10 most boron-sensitive and 10 most B-insensitive accessions of Brassica napus and B. rapa in a replicated field trial at the Muck Crop Research Station in 2015. Different letters above the bars indicate differences based on Tukey's test at $P < 0.05$.

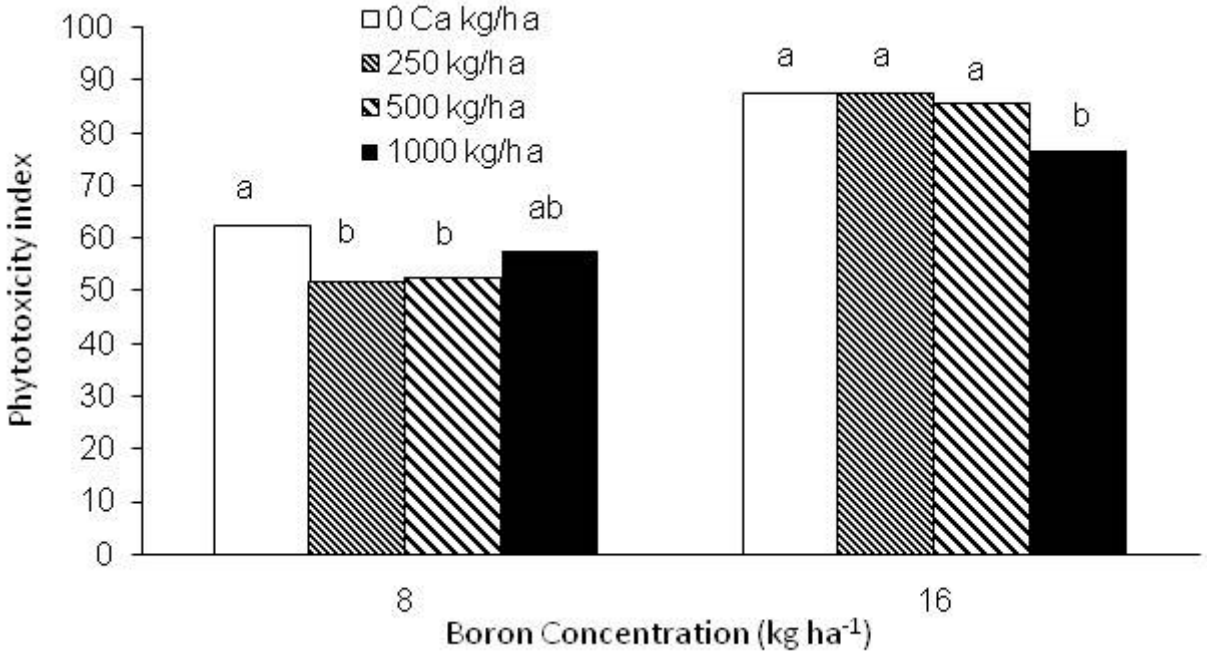

Figure 2

Effect of the rate of calcium (Ca) applied to soil on the phytotoxicity of boron on a B-sensitive line of canola in a growth room study.

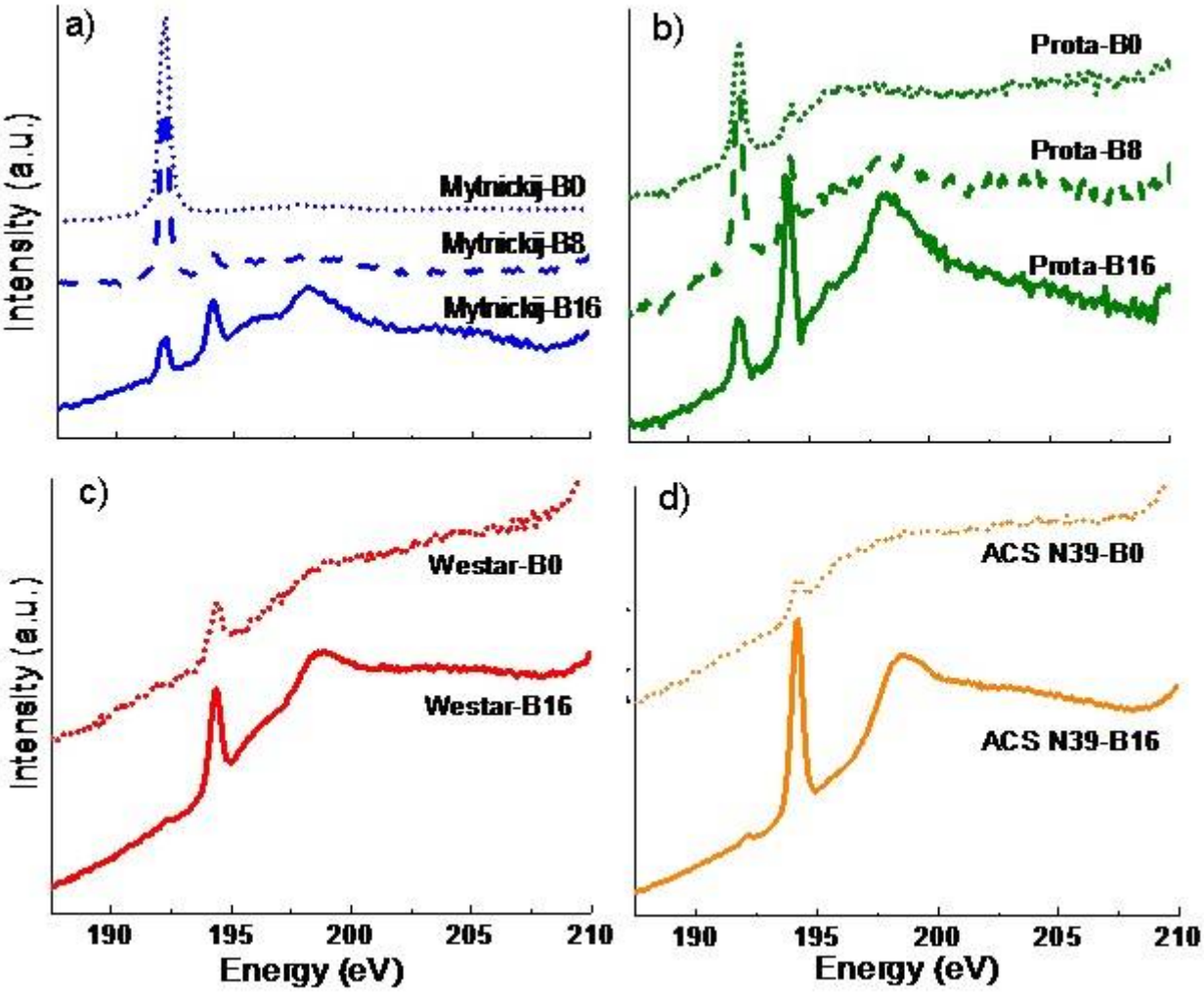

Figure 3

Boron K-edge XANES spectra collected on roots of the B-insensitive accessions A) Mytnickij and B) Prota, and the B-sensitive accessions C) Westar and D) ACS N39 treated with 0, 8 or 16 kg B ha-1 (B0, B8 and B16, respectively).

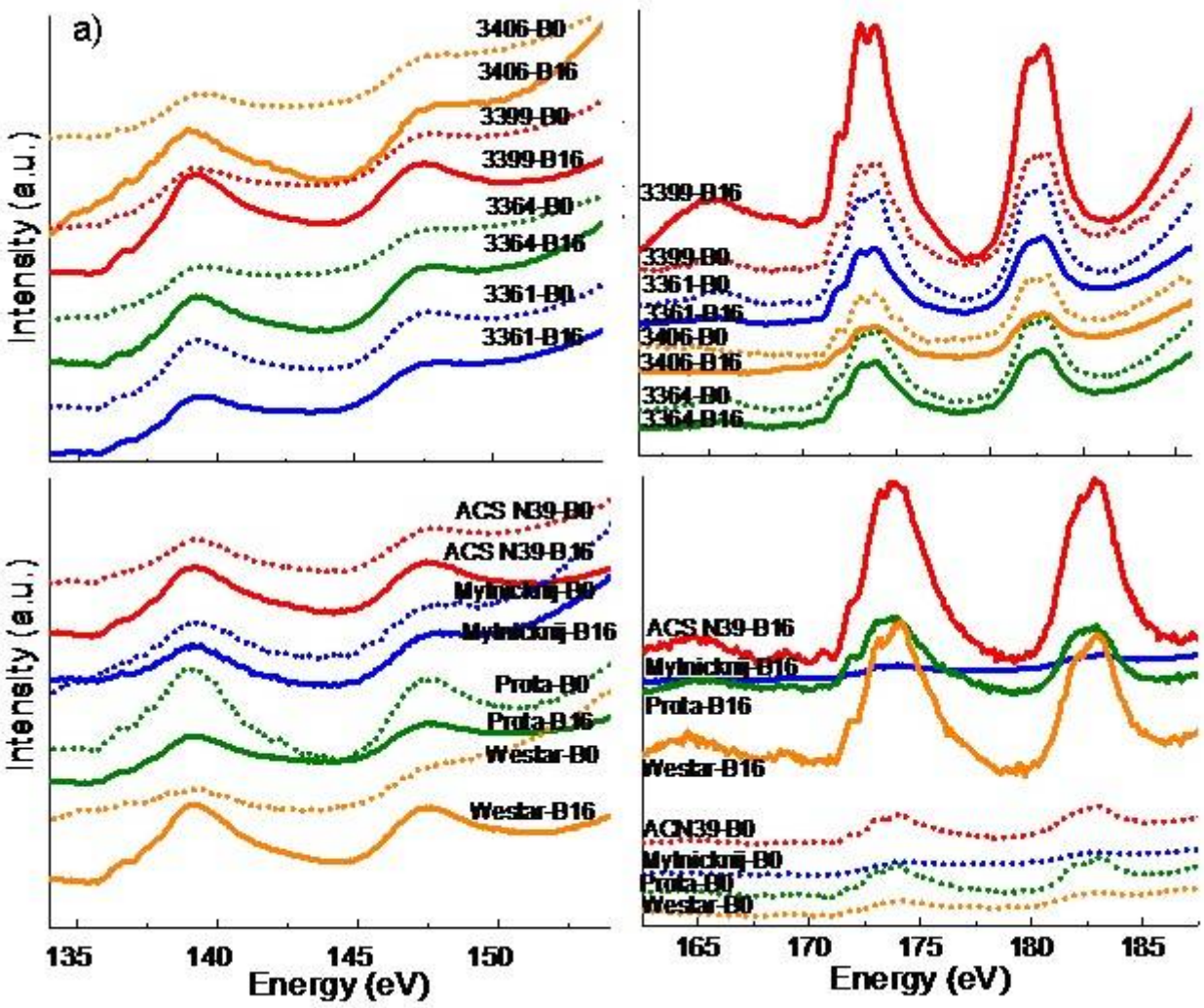

Figure 4

Boron K-edge XANES spectra collected on leaves of the B-insensitive accessions A) Mytnickij and B) Prota, and the B-sensitive accessions C) Westar and D) ACS N39 treated with 0, 8 or 16 kg B ha-1 (B0, B8 and B16, respectively).

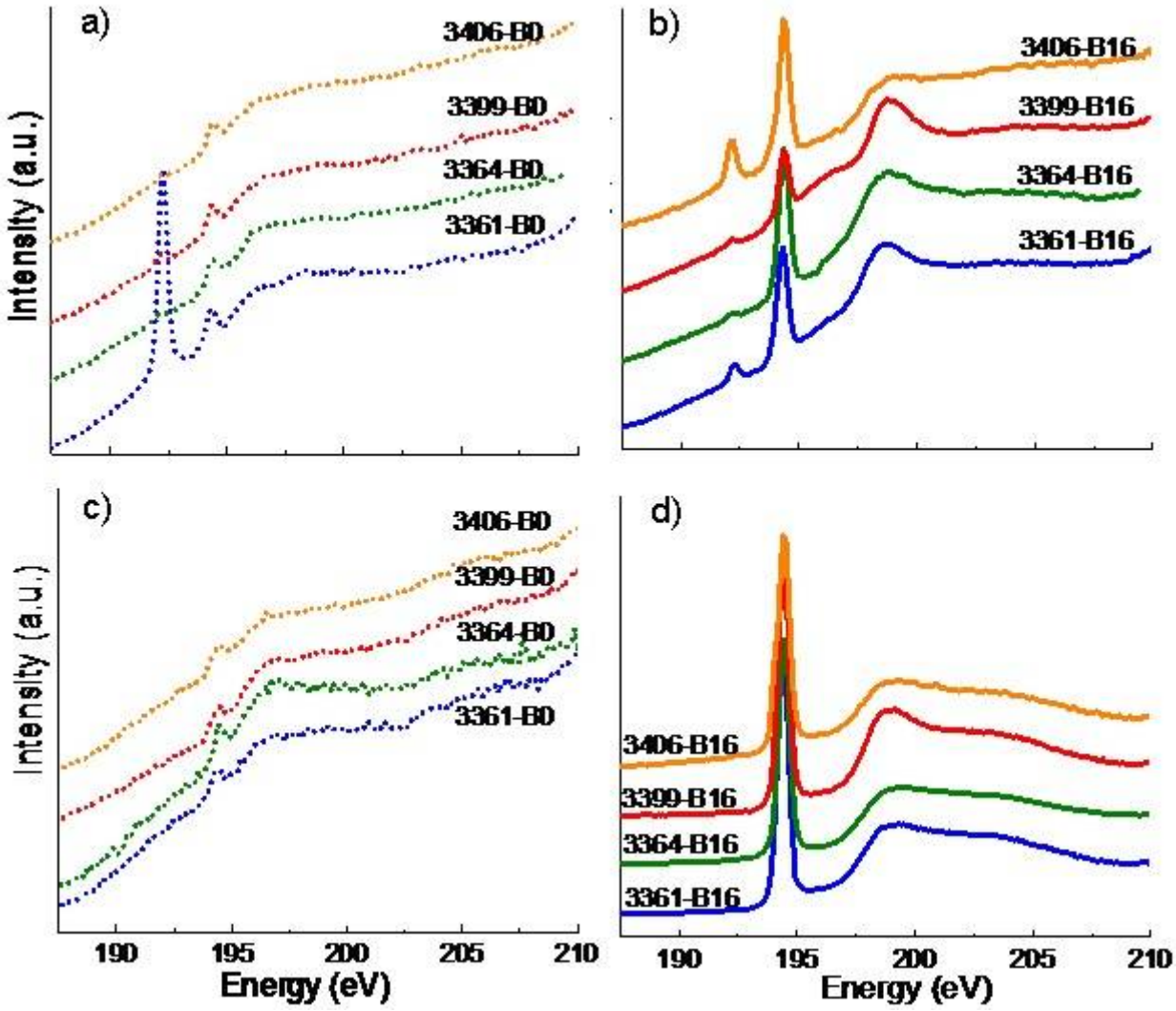

Figure 5

Boron K-edge XANES spectra collected on roots of the B-sensitive accessions SRS3399 and 3406 and B-insensitive accession SRS 3361 and 3364, and treated with 0 or 16 kg B ha-1 (B0 and B16, respectively).

Supplementary Files

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