Letter

Boron: an essential element for vascular plants

A comment on Lewis (2019) 'Boron: the essential element for vascular plants that never was'

Introduction

Recently, Lewis (2019) claimed in a provocative Viewpoint that boron (B) is not essential for plants, and negated its compliance with the third criterion for essentiality, namely that essential elements have metabolically direct effects. He proposed that B has always been solely toxic but has the capacity to quench the toxicity of phenolics by formation of non-toxic complexes, suggesting that B deficiency phenotypes are misjudged phenolic toxicity symptoms.

This Letter counters the Viewpoint by critically examining the experimental evidence that B plays a unique role for plant cell wall function and is thus essential for vascular plant life. Moreover, it challenges the postulations made by Lewis regarding a reciprocal mitigation of boron and phenolics toxicity.

After almost 100 years of research on B it is certain that the role of B in plants and animals has not yet been exhaustively clarified. In this regard, the provocative Viewpoint by Lewis (2019) is highly appreciated as it will spur renewed discussion on possible B functions and important roles as a consequence of its interactions with cellular metabolites. The authors of this Letter do not, however, support the foundational contentions of Lewis and would like to offer this rebuttal to many of his conclusions.

Despite the controversial interpretation of available evidence, we indeed agree that further experimental efforts and scientific discussions are needed to reconcile our imperfect knowledge on B functions and its interaction with the phenol metabolism. We are however concerned that the provocative suggestion that B is not an essential plant nutrient will have a detrimental impact on agriculture as B is one of the most frequently deficient and actively managed micronutrients in crops and B fertilization is critical for achieving optimal agricultural productivity.

Boron

Boron is a unique element since it can form strong complexes with different molecules carrying *cis*-diol groups in appropriate spatial configurations (Makkee *et al.*, 1985). Complexes of B with apiose, ribose, nicotinamide adenine dinucleotide (NAD), *S*-adenosyl methionine (SAM), phenolics, mannitol, sorbitol, sucrose, amino acids and larger molecules such as glycopeptides and glycoproteins have been detected at least *in vitro* (Loomis & Durst, 1992; Power & Woods, 1997; Blevins & Lukaszewski, 1998; Sparbier *et al.*, 2006; Nielsen & Meacham, 2011).

Following the work of Warrington (1923), demonstrating that vascular plants cannot reproduce without B, it was listed as an essential element for vascular plants. If vascular plants face B-deficient conditions, a variety of symptoms such as a reduced root and shoot growth and a strongly impaired fertility are observed. The impact of B deficiency is specifically observed in meristematic tissues within minutes of B removal (Lovatt, 1985), with recent evidence suggesting a critical role in root and shoot apical meristem in the quiescent zone (Shimotohno *et al.*, 2015; Poza-Viejo *et al.*, 2018). The rapidity and tissue-specific nature of B deficiency suggest that B is required preferentially in meristematic tissues (Brown & Hu, 1997; Brown *et al.*, 2002). Any purported additional function of B in vascular plants, therefore, must also satisfy this common observation.

Boron fulfils all requirements for essentiality

The term essential mineral element (or mineral nutrient) was originally proposed by Arnon & Stout (1939). These authors suggested that three criteria must be met for an element to be considered essential:

(**1**) A given plant must be unable to complete its lifecycle in the absence of the element.

(**2**) The function of the element must not be replaceable by another element.

(**3**) The element must be directly involved in plant metabolism.

According to this definition, an element which partly substitutes for another element or which has simply positive effects on plant growth, for example in correcting unfavorable conditions of the growth medium may not be described as an essential element but rather as a beneficial one. Lewis (2019) seems to agree that plants are unable to complete their lifecycles in the absence of B and that B cannot be replaced by another element and therefore meets criteria 1 and 2 of the earlier definition, but he challenges the third criterion and suggests that the effects of B are not directly involved in the plant metabolism.

Indeed, the third criterion of essentiality was previously challenged (Epstein & Bloom, 2005), since it makes the list of essential elements dependent on complete understanding of nutrient functions. Regarding B, possibly millions of farmers since the last century around the world have observed that plants do not grow without sufficient supply of B, even before a 'direct involvement in plant metabolism' was proven. However, at least since the unambiguous function of B in cell wall metabolism was established, the third criterion for essentiality is also met. Indeed, many other established essential elements also function by virtue of their binding with metabolites.

Cell wall integrity: the primary metabolic role of boron in vascular plants

Lewis' statement that 'there is no consensus about its primary role' and 'that no primary role has been agreed despite the long history of research surely indicates that it does not have one', is erroneous. Quite the contrary, there is nowadays wide consensus amongst plant biologists that the cross-linking of RG-II molecules by B is an essential function of B in higher plants. The formation of borate di-ester crosslinks with two RG-II monomers is essential for a proper formation and stabilization of primary cell walls in vascular plants (O'Neill *et al.*, 1996, 2001, 2004; Matoh, 1997). Moreover, it is established that B-dependent cell wall integrity is important for a proper signal transduction from the apoplast to the cytoplasm and as such is critical for cellular function (Wolf *et al.*, 2012). Since the cross-linking of two RG-II molecules has been unambiguously demonstrated, and clearly is a direct molecular interaction between B and the RG-II molecules, the remaining question is whether the cell wall formation is part of the plant's metabolism or not. The answer to this question is also unequivocally yes: first, B-mediated RG-II dimerization seems to occur intracellularly in the phragmoplast of actively dividing cells (Chormova *et al.*, 2014; Dumont *et al.*, 2016; Zhou *et al.*, 2017), and second, cell wall integrity is essential for growth, development and reproduction. The Viewpoint of Lewis (2019) does not provide any argument for why this most direct and well-described function of B in cell wall formation is not considered a part of the plant's metabolism. There is also no evidence to suggest that phenol metabolism plays any direct role in the formation of the critical B–RG-II crosslink in plant cell walls. Indeed, the primary article cited by Lewis, namely Hull (2002), does not question that B is essential for cell wall structure but merely questions whether additional functions remain to be discovered. This is a view that is shared by many authors (Brown *et al.*, 2002; Marschner, 2012), as other B ligands may exist in plants (Wimmer *et al.*, 2009; Reguera *et al.*, 2010; Voxeur & Fry, 2014; Chormova & Fry, 2016). However, the uncertainty about additional functions of B does not negate its established role in the cell wall function.

Metabolic multi-functionality is common amongst plant micronutrients and does not contradict the essentiality of boron

In his Viewpoint Lewis states that evidence of the multifunctional nature of B [sic] 'contrasts with the roles of other essential micronutrients that generally have restricted, specific roles' (Lewis, 2019). This statement is not only incorrect (most essential elements are indeed multifunctional), but it is also internally inconsistent since one cannot simultaneously argue there is no essential function for B while concluding that the multifunctional nature of B excludes essentiality.

Micronutrient toxicity does not contradict essentiality

Further, Lewis stated that '. . . boron is, and always has been, potentially toxic . . .', '. . . that it is not essential in the conventional sense because it is always toxic and so cannot have a primary role . . .' and '. . . since a toxic element cannot have deficiency symptoms . . .' (Lewis, 2019). This conclusion, however, overlooks the fundamental nature of substances as related by Paracelsus in the fifteenth century: 'All substances are poisons; there is none which is not a poison. The right dose differentiates a poison and a remedy' (Peukert, 1965). Clearly, the other essential micro-elements (zinc (Zn), manganese (Mn), nickel (Ni), iron (Fe), copper (Cu)) are also potentially toxic, and many examples can be cited, for example Cu is essential in numerous Cu-metalloenzymes and yet the cytosolic free Cu concentration in yeast is restricted to 10^{-18} M to prevent deleterious cytotoxic effects of free Cu (Rae *et al.*, 1999). The suggestion that the potential for toxicity excludes the potential for essentiality, is not supported. Indeed, it is well known that other micro-elements, which are toxic at higher concentrations, still can induce deficiency symptoms at low bioavailable concentrations and this is nicely illustrated by the so-called 'growth response curve' representation which demonstrates the relationship between plant growth and nutrient supply.

In addition, if B was only toxic, one would wonder why the bryophyte *Physcomitrella patens*, which is lacking RG-II, does not suffer from B toxicity despite extremely high levels of free 'toxic' B in the cell wall. *Physcomitrella patens* has cellular B concentrations (G. P. Bienert, unpublished results) and total B contents in the cell wall (Matsunaga *et al.*, 2004) comparable to vascular plants, but 95% of the cell wall B is present as free B without resulting in toxicity symptoms. *Physcomitrella patens* would represent a suitable model plant system to further address this question.

Correlation of boron and phenols does not imply causality

Lewis states: '. . . that low endogenous concentrations of boron are correlated with an enhanced presence of potentially toxic phenolics . . .' (Lewis, 2019).

The main argument of Lewis (2019) against the essentiality of B is that its only crucial role in the plants' metabolism is restricted to the ability to form complexes with diverse phenolic compounds, thereby quenching the cytotoxic effects of both species. To our knowledge, no B–phenol complexes have so far been analytically identified *in vivo*. There is evidence that the B nutritional status of vascular plants influences the phenol metabolism and vice versa, but the specific nature of these interactions is not established (Marschner, 2012). There are also reports of no effects of B fertilization on phenolics, while phenoloxidase and polyphenoloxidase concentrations were increased (Ruuhola *et al.*, 2011). In conifers, B fertilization has reduced tannins to a greater extent than low-molecular weight phenolics, suggesting a shift to the more mobile forms (Rummukainen *et al.*, 2007, 2013). This is not consistent with a role of B in phenol detoxification. Several studies describe, in mostly one-time-point measurements, an increase in phenolic quantities following mostly long-term exposure to growth inhibiting low B levels, though none of them distinguishes between non-complexed and B-complexed phenolics. To establish or hypothesize a function of B in the detoxification of phenolics would require knowledge of the organ-specific levels of free

phenolics (not complexed with B). Such evidence is currently lacking.

In addition, an observed inverse correlation between B and (total) phenolic levels, cited by Lewis, does not provide evidence of an essential and functional relationship. Over the past 90+ years of B research, many researchers have observed changes in metabolites or metabolism and proposed that these changes suggest a specific primary function for B. However, the rapid occurrence of growth inhibition upon abrupt imposition of B deficiency has complicated B research as the near immediate growth perturbations caused by B deficiency rapidly disrupt all manner of metabolism (Brown *et al.*, 2002). The correlation between B and phenolic levels cited by Lewis can very clearly be a result of such a growth perturbation and subsequent re-ordering of cellular carbon flows and does not provide evidence for an essential and functional relationship.

A causal relationship between B and phenolics is also not supported by the importance of B for the formation of functional heterocysts in cyanobacteria like *Anabaena*, most likely by tightening the hull and providing an oxygen barrier to protect the nitrogenase (Bonilla *et al.*, 1990; Bolanos *et al.*, 1994). Detoxification of phenolics by B as 'the' mode of action seems to be far-fetched and a highly unlikely mechanism. In addition, the B requirement during the early phases of embryonic development of animals, and the presence of a B transporter in various mammalian species all point to some specific roles of B in the animal metabolism which are definitely independent from the need to quench phenol toxicity and also cell wall stability (Fort *et al.*, 1999; Hunt, 2007; Parker & Boron, 2013).

Does subcellular partitioning of boron support quenching of phenol toxicity?

Lewis states that '... physical sequestration of free or complexed boron in vacuoles and/or apoplast also enhances the maintenance of the uninhibited cytoplasmic metabolism' (Lewis, 2019). This suggests that B toxicity is mitigated by physical sequestration of free or complexed B in vacuoles and/or the apoplast. However, to the best of our knowledge, experimental information on vacuolar B concentrations or molecularly characterized B-complexes within vacuoles does not exist and studies on the kinetics of B transport across plant cell membranes experimentally or by modeling suggest only a marginal amount of internal B complexation (Stangoulis *et al.*, 2001; Shimotohno *et al.*, 2015). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry which was used to identify non-cell wall bound B in different plant species, also did not detect B–phenol complexes (Stangoulis *et al.*, 2010).

Is there sufficient chronological and quantitative evidence to support the contention that boron–phenol complexes are responsible for boron deficiency effects?

Additionally, an in-detail analysis of the dynamic development of B and phenol concentration patterns (the chronology of effects) is also still missing. Studies with sequential harvests suggest that B and phenol concentrations are not closely linked. Several studies describe typical B deficiency symptoms such as root growth inhibition and root hair elongation within 24 hours or at least within the first days of B withholding (González-Fontes *et al.*, 2016; Pommerrenig *et al.*, 2018; Poza-Viejo *et al.*, 2018), while increases of phenolics or alterations in phenylalanine ammonia-lyase (PAL) or polyphenol oxidase (PPO) activities have so far only been reported after much longer times (e.g. Camacho-Cristóbal *et al.*, 2002: 5–7 days). If B deficiency is only a consequence of phenolic toxicity, then a coincident change in free phenolics levels with imposition of reduced B would be expected. Also, to our knowledge, no reports are available describing the opposite effect, namely that phenolic levels are reduced when B is re-supplied after B was withheld, or that physiologically relevant toxic phenolic effects are mitigated under surplus B supply.

However, the adaptation of phenylpropanoid profiles to changing environmental conditions is highly dynamic with tightly controlled temporal and spatial patterns (Weisshaar & Jenkins, 1998; Jenkins *et al.*, 2001). In addition, changes in phenolic compounds in response to high light intensities, cold or combinations of both stresses in Arabidopsis, tobacco, sunflower, sugar beet and barley were species specific. For example, cinnamic acid conjugates were specifically affected in sunflower, whereas particularly flavonoid compounds (kampferol and quercetin derivates) were responsive in other species. Genotypic differences in phenylpropanoid profiles were reported in sugar beet, barley and Arabidopsis mutants (Petridis *et al.*, 2016). Even though we cannot exclude interaction of phenolics with B under specific conditions, it seems highly unlikely that B concentrations and distribution would follow these rapid changes in soluble phenylpropanoids.

The inconsistent nature of B–phenolic correlation is also demonstrated by contrasting results from *Olea europaea* plants grown either under B-deficient conditions in a growth chamber or in the field. While leaf B concentrations were similar and identical visible B deficiency symptoms were observed in the low-B plants (Liakopoulos & Karabourniotis, 2005), phenolic compounds only accumulated in leaves of growth chamber grown plants but not in leaves of field grown plants. The differences may be related to the effects of UV and visible radiation on phenolics, which can be much larger than that of B nutrition (Close & McArthur, 2002). In a different study, phenol and especially condensed tannin concentrations were elevated in B-deficient *Picea abies* seedlings maintained under growing-season conditions, but a simulated autumn increased the tannin concentrations much more and the B effect disappeared (Rummukainen *et al.*, 2007). All these results indicate that the B deficiency symptoms cannot be explained by the total amount of phenolics within the leaves and contradicts the hypothesis of the Viewpoint.

Moreover, evidence for a role of B in the detoxification of phenols, or the reciprocal effect of phenols on B, is lacking. To date, no systematic analysis has been performed to compare in detail the toxicity of distinct B or phenolic levels with comparable B–phenolic complex levels. Even without such necessary biochemical assays, simple stoichiometric analysis of reported cellular B and potential B-complexing molecule concentrations suggests that B

concentrations are not sufficient to influence phenol metabolism through complexation (Marschner, 2012, p. 236). Phenolic concentrations range from 5000 to 50 000 micrograms of phenols per gram of dry weight of plant tissues (Swanson, 2003) compared to only 2 to 100 micrograms of B per gram of dry weight of plant tissues, typical of most plant species (Marschner, 2012). Assuming, for the sake of simplicity, an average molecular weight of 100 g mol^{-1} for simple phenolics, this would result in an *c.* 25-fold surplus of phenolics compared to total B in plant tissues under sufficient B supply. Given that large quantities of B are complexed in plant cell walls, then free B levels would be even lower, and certainly far too low to influence phenol metabolism through complexation under adequate B nutrition.

Several studies demonstrated that an increasing supply of B resulted in enhanced plant growth, which according to the proposal of Lewis would be solely a result of a higher capacity to take toxic phenolics away from the primary metabolism. However, experimental data showed that concentrations of B and phenolics do not linearly correlate with increasing B level supplies as the plant growth does (Ruiz *et al.*, 1998).

Lewis (2019) also argues that phenolics are the only likely 'neutralizing agents' for excess B, apparently overlooking the documented complexes of B with a range of other molecules. Certain plant species contain very high levels of potential B-binding carbohydrates such as sorbitol, which may be present, for example in peach (*Prunus persica*) at higher than 0.5 M (Moing *et al.*, 1992), which is > 500 times the typical B levels. Under these conditions, complexes of B with phenolics would be unlikely unless the binding affinity of the respective compounds is very much higher than that of B–sorbitol. If the reciprocal correlation of B and phenolics was correct, then one would expect phenolic levels in polyol producing species to be much lower than in species not producing sorbitol and this is not the case.

As another example, the concentration of B required for graminaceous monocots (e.g. barley or wheat) is 10- to 15-fold lower than for dicot plants. However, there is no evidence to suggest these species have a commensurately lower phenol concentration as would be suggested by Lewis' hypothesis. The same is true for within-species differences in B demand, where studies using QTL (quantitative trait locus) approaches also failed to find links between the B demand and physiological consequences of a diverging phenylpropanoid metabolism (Schnurbusch *et al.*, 2010; Zhang *et al.*, 2014; Hua *et al.*, 2016).

Collectively all these results rather indicate that B deficiency symptoms are not solely an expression of phenol toxicity, but rather an orchestrated response. This is supported also by recent results indicating that some symptoms of B deficiency can be alleviated by altering hormone concentrations (using plant mutants or drugs), or reactive oxygen species (ROS) inhibitors (Camacho-Cristóbal *et al.*, 2015; Poza-Viejo *et al.*, 2018). In the absence of information on abundances and organ level localization of free phenolics, it is thus not sound to conclude that '... boron's inherent, concentration-dependent toxicity is wholly or largely ... achieved by combination of the offending element with commensurate, stoichiometric amounts of appropriate phenolics' (Lewis, 2019).

In conclusion: (1) the proven function of B for RG-II-dimerization, (2) the rapid response reaction(s) found very shortly after withholding B from the nutrient solution, and (3) the unfavorable stoichiometric ratios between B and phenylpropanoids, all contradict the hypothesis that phenylpropanoids are crucial determinants for B detoxification and that the sole role of B is to prevent toxic effects of phenolic substances.

Conclusions

While the Viewpoint of Lewis (2019) is certainly provocative and as such will stimulate research to further clarify the still incomplete understanding of the role of B in plants or animals, we find the hypothesis that B is not essential for higher plants is untenable, as neither convincing theoretical nor experimental data to support this thesis has been presented. The widely accepted and replicated finding that B cross-links RG-II and that the RG-II cross-link is essential for normal cell wall function and plant growth, represents a clear demonstration of essentiality. The suggestion that B–phenol interactions may occur and may be essential for plant life does not diminish the demonstration of a direct role of B in metabolism through its critical role in the cell wall. While we encourage experimental efforts to elucidate B functions, it is critical that the supply of B fertilizers in agriculture is maintained and that growers continue to use B compounds to maintain crop yields where necessary.

Before all previous studies on B are re-evaluated with respect to confounding effects of phenolics because they are '... studies of abnormal metabolism caused by the adverse inhibitory effects of miscellaneous phenylpropanoids present in excess of the binding capacity of endogenous boron' (Lewis, 2019), and before future work is complicated by additional measurements of all kinds of phenolics, we alternatively suggest that some of the proposed mechanisms are first tested experimentally by conducting basic assays, such as: (1) identification of B-interacting phenolics, for example by affinity purification using Amberlite IRA743 resin or boronic acid resins (Wimmer *et al.*, 2009; Reguera *et al.*, 2010), or alternatively by means of liquid chromatography coupled with mass spectrometry, (2) temporal responses of plants to stresses inducing phenolics including measurements of free and bound B, (3) temporal responses to low B supply including measurements of (free and B-complexed) phenolics, (4) experiments using double gradients of boric acid and commercial phenolics to test whether complexation inhibits toxicity of both components, (5) combined omics approaches, and (6) measurements made in specific plant cells and tissues, particularly in meristems.

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Author contributions

MAW and GPB led the writing of the manuscript and wrote the first draft. All other authors contributed to the writing of the manuscript.

ORCID

Isidro Abreu https://orcid.org/0000-0002-1647-9883
Elias Bassil https://orcid.org/0000-0001-8109-5758
Richard W. Bell https://orcid.org/0000-0002-7756-3755
Gerd P. Bienert https://orcid.org/0000-0001-9345-4666
Manuela D. Bienert https://orcid.org/0000-0001-5948-3789
Patrick H. Brown https://orcid.org/0000-0001-6857-8608
Bernard Dell https://orcid.org/0000-0001-8623-6425
Toru Fujiwara https://orcid.org/0000-0002-5363-6040
Heiner E. Goldbach https://orcid.org/0000-0003-0731-8057
Tarja Lehto https://orcid.org/0000-0003-2281-6409
Hans-Peter Mock https://orcid.org/0000-0002-3983-3099
Monika A. Wimmer https://orcid.org/0000-0002-7456-5667
Nicolaus von Wirén https://orcid.org/0000-0002-4966-425X

Monika A. Wimmer[1]*, Isidro Abreu[2,3], Richard W. Bell[4], Manuela D. Bienert[5], Patrick H. Brown[6], Bernard Dell[4], Toru Fujiwara[7], Heiner E. Goldbach[8], Tarja Lehto[9], Hans-Peter Mock[5], Nicolaus von Wirén[5], Elias Bassil[10] and Gerd P. Bienert[5]*

[1]Department Quality of Plant Products, Institute of Crop Science, University of Hohenheim, 70599 Stuttgart, Germany;
[2]Centro de Biotecnología y Genómica de Plantas (UPM-INIA), 28223, Pozuelo de Alarcón, Madrid, Spain;
[3]Departamento de Biotecnología-Biología Vegetal, Escuela Técnica Superior de Ingeniería Agronómica, Alimentaria y de Biosistemas, Universidad Politécnica de Madrid, 28040, Madrid, Spain;
[4]Agriculture Discipline, College of Science, Health, Engineering and Education, Murdoch University, Perth, WA 6150, Australia;
[5]Department of Physiology and Cell Biology, Leibniz Institute of Plant Genetics and Crop Plant Research, 06466 Gatersleben, Germany;
[6]Department of Plant Sciences, University of California-Davis, Davis, CA 95616, USA;
[7]Department of Applied Biological Chemistry, The University of Tokyo, Tokyo 113-8657, Japan;
[8]Institute of Crop Science and Resource Conservation (INRES), University of Bonn, 53115 Bonn, Germany;
[9]School of Forest Sciences, University of Eastern Finland, 80110, Joensuu, Finland;
[10]Horticultural Sciences Department and Tropical Research Education Center, University of Florida, Homestead, FL 33031, USA
(*Authors for correspondence: tel +49 71145922463, email m.wimmer@uni-hohenheim.de (MAW); tel +49 394825385, email bienert@ipk-gatersleben.de (GPB))

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