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ROOT PLASTICITY IN TWO CONTRASTING BORON TOLERANT TOMATO GENOTYPES UNDER BORON EXCESS

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"Although nature commences with reason and ends in experience it is necessary for us to do the opposite that is to commence with experience and from this to proceed to investigate the reason."

Leonardo da Vinci

(15th April 1452 – 2nd May 1519)

A Pietro, mio marito...

Per l'incoscienza, l'allegria, la testardaggine e l'Amore

Che ogni giorno mi regala e ricorda!!!

CONTENTS

SINTESI

Il boro (B) è un micro-elemento essenziale per la crescita delle piante, e l'importanza della sua applicazione in sistemi colturali intensivi è ben riconosciuta. Esso è infatti fondamentale nella costituzione della parete cellulare delle piante e quindi nei processi di formazione ed allungamento delle radici e dei germogli, nonché di distensione delle foglie. Appare altresì ormai certa la sua funzione nel garantire l'integrità delle membrane cellulari.

Ciò nonostante, il B risulta tossico se presente a concentrazioni elevate in suoli ricchi di B o a causa di un'eccessiva concimazione e/o irrigazione con acqua ricca di questo elemento. In alcune regioni del Mediterraneo, la contaminazione dei terreni e delle acque irrigue da B (con concentrazioni circa 15 mg/L) rappresenta una seria minaccia per le colture e per la salute umana.

Il B viene assorbito dalle radici come acido borico e tende ad accumularsi nelle foglie mature, specialmente ai loro margini, in quanto è trasportato lungo il sistema di traspirazione e si accumula alla fine del flusso traspirazionale. Il tipico sintomo di tossicità da B è la necrosi marginale fogliare. Tuttavia, in altre specie (ad esempio mela, pesca e mandorla), il B può essere rimobilizzato attraverso il floema da zuccheri quali mannitolo e sorbitolo, in grado di legare l'acido borico.

La tolleranza della pianta alla tossicità da B è specie-specifica ed è generalmente associata alla capacità di limitare il suo assorbimento e/o trasporto attraverso meccanismi di esclusione e/o di efflusso attivo dalle radici. In *Arabidopsis*, la tolleranza al B è associata alla presenza di canali BOR, che sono responsabili della sua estrusione dal citoplasma. Due famiglie geniche sembrano regolare l'assorbimento ed il trasporto del B nelle piante: i) BOR1, un trasportatore *efflux-type* coinvolto nel caricamento del B nello xilema; e ii) NIP, le proteine intrinseche nodulin-like, canali candidati per il trasporto di membrana. Recenti lavori hanno dimostrato che entrambi questi canali, BOR 1 e NIP, hanno un ruolo fondamentale in condizione di carenza di B nelle piante mentre BOR4, un trasportatore B *efflux* dalle radici al terreno, è considerato il maggiore responsabile nel conferire tolleranza al B.

Sebbene i tipici sintomi della tossicità da B si manifestano a carico delle foglie, anche la radice appare un bersaglio altamente sensibile all'eccesso di B in quanto la sua crescita risulta notevolmente ridotta. E' stata inoltre recentemente evidenziata l'importanza della morfologia radicale nel conferire tolleranza al B in diverse specie quali frumento, orzo, riso e pomodoro, sottolineando il ruolo chiave della radice in risposta alla tossicità da B.

In tale ottica, il lavoro di tesi di dottorato ha posto particolare attenzione all'apparato radicale, nella sua forma e funzione, focalizzando l'attenzione sulle risposte morfo-fisiologiche e molecolari ad elevate concentrazioni di B in pomodoro. Il pomodoro è una delle specie ortive più importanti nel bacino del Mediterraneo sia per superficie coltivata sia per produttività.

L'attività di ricerca si è prefissata 3 obiettivi fondamentali:

1) Effetti morfologici, fisiologici e molecolari di lunghe e brevi esposizioni ad elevate concentrazioni di B sulla forma e funzione dell'apparato radicale di due genotipi di pomodoro con diversa sensibilità ad eccesso di B;
2) Risposte antiossidanti a brevi esposizioni ad eccesso di B in due genotipi di pomodoro con diversa sensibilità ad eccesso di B;
3) Risposte all'eccesso di B in pomordoro: il ruolo dell'innesto e della morfologia radicale.

Il primo obiettivo è stato quindi quello di studiare le risposte morfo-fisiologiche e molecolari di due ibridi di pomodoro, Ikram e Losna, caratterizzati da diversa sensibilità all'eccesso di B, dopo lunga e breve esposizione ad elevate concentrazioni di questo elemento. In particolare, è stato realizzato uno studio integrato delle risposte di forma e funzione della radice valutando altresì le interazioni di questo stress con il nitrato, nutriente essenziale per la crescita e lo sviluppo delle piante.

È stato inizialmente condotto uno *screening* fenotipico per la tolleranza al B esponendo i due genotipi ad elevate concentrazioni di B per 7 giorni (trattamento a lungo-termine). Sul materiale vegetale è stata valutata l'espressione dei sintomi di tossicità a livello fogliare ed il contenuto di clorofilla. E' stata inoltre analizzata la morfologia radicale ed infine è stato determinato il contenuto in B nei tessuti radicali e fogliari. I risultati hanno permesso di evidenziare una maggiore tolleranza di Losna all'eccesso di B, rispetto ad Ikram. In particolare, Ikram mostrava evidenti sintomi di tossicità fogliare, confermati da un ridotto contenuto in clorofilla e maggiore concentrazione interna di B rispetto a Losna, in entrambe gli organi fogliare e radicale. Ancora più interessanti sono stati i risultati sulla morfologia radicale, che hanno confermato una maggiore capacità di adattamento di Losna rispetto ad Ikram all'eccesso di B. Infatti, Losna, oltre a mantenere inalterata la lunghezza radicale in presenza di alte concentrazioni di B, mostrava un aumento del rapporto di lunghezza radicale (RLR), importante indice di potenzialità della radice per l'acquisizione delle risorse. È stata inoltre effettuata l'analisi delle componenti di 'allocazione' e 'strutturali' dell'RLR quali il rapporto di massa radicale (RMR), la finezza e la densità di tessuto. I risultati hanno evidenziato che Losna è in grado di investire sull'apparato

radicale mantenendo elevata la sua crescita anche in presenza di elevate concentrazioni di B mentre Ikram mostrava una maggiore densità di tessuto, componente strutturale strettamente correlata al processo di lignificazione della radice.

Successivamente, è stato valutato se il diverso comportamento osservato nei due genotipi dopo lunga esposizione all'eccesso di B (0, 320 e 640 μM) fosse confermato anche dopo brevi esposizioni (48 h). Inoltre, poiché è noto che cambiamenti nella forma (morfologia radicale) sono generalmente accompagnati da cambiamenti funzionali della radice (assorbimento dei nutrienti) sono stai studiati gli effetti fisiologici e molecolari dell'eccesso di B sull'assorbimento del nitrato. I risultati hanno evidenziato che tutti i trattamenti con B inibivano significativamente l'assorbimento del nitrato in Ikram, mentre in Losna tale inibizione si osservava solo alla concentrazione più elevata. Simile pattern mostrava anche l'attività della pompa H^+-ATPasica, enzima strettamente funzionale alle attività di assorbimento del nitrato, in entrambe i genotipi. L'analisi dei geni relativi all'assorbimento del nitrato (NTR2.1, NAR2.1) ed alla pompa protonica (LHA1, LHA8) erano in linea con i dati biochimici e fisiologici ottenuti, evidenziando una maggiore inibizione di espressione in Ikram rispetto a Losna.

Successivamente, sono state effettuate nei due genotipi analisi di espressione dei geni in grado di conferire tolleranza a tale stress, dopo breve esposizione all'eccesso B. Questo avrebbe permesso di capire quanto precoce fosse l'espressione della tolleranza in Losna e quali geni ne fossero responsabili. E' noto infatti che la tolleranza delle piante alla tossicità del B è essenzialmente legata alla loro capacità di ridurre l'assorbimento radicale del B grazie a meccanismi di esclusione e di efflusso attivo dalle cellule radicali. In tale ottica, sono state valutate le espressioni dei geni codificanti per i trasportatori trans-membrana dell'acido borico nelle piante (BOR4, BOR1, NIP5;1).

I risultati ottenuti hanno evidenziato una maggiore espressione del gene BOR4, responsabile dell'efflusso del B da parte delle cellule radicali nel mezzo esterno in Losna rispetto a Ikram. Al contrario, i geni BOR1 e NIP5;1 mostrano un espressione ridotta in entrambi i genotipi, confermando il loro ruolo fondamentale in condizione di carenza da boro ma non di eccesso.

Questi risultati suggeriscono che uno dei possibili meccanismi in grado di conferire tolleranza al B in Losna implica un efflusso dell'anione borato da parte delle cellule radicali. Questo potrebbe supportare i dati di minore contenuto di B riscontrati in Losna rispetto ad Ikram. Recentemente, è stato postulato che l'efflusso attivo di B attraverso i trasportatori BOR-type necessita di un input di energia che guida il gradiente concentrazione attraverso la membrane. Poiché lo ione che guida i

sistemi di co-trasporto attraverso la membrana plasmatica è solitamente lo ione H^+, semplici esperimenti elettrofisiologici in presenza di stimolatori o inibitori della pompa H^+-ATPasi sono stati eseguiti in radici di entrambe i genotipi per meglio comprendere il meccanismo di tolleranza. I risultati hanno fortemente suggerito il coinvolgimento di un efflusso elettrogenico di protoni che avviene dopo trattamento con lo ione borato. In particolare, Losna mostrava una maggiore iperpolarizzazione di membrana (potenziale di membrana più negativo) rispetto ad Ikram in risposta ad alte concentrazioni di B. Tale effetto era evidente anche quando le radici di pomodoro erano esposte contemporaneamente a B e nitrato o cumarina, sostanze in grado di determinare un'iperpolarizzazione della membrana plasmatica. Questi dati suggerivano che la tolleranza al B in pomodoro era dovuta ad un efflusso borato guidato da efflusso di protoni. Tuttavia, per comprendere se questo efflusso di ioni H^+ fosse H^+-ATPasi dipendente, è stato utilizzato il vanadato, un forte inibitore dell'attività della pompa H^+-ATPAsi dipendente. I risultati hanno evidenziato che in Losna la tolleranza all'eccesso di B è dovuta ad un efflusso di B accompagnato da un contemporaneo efflusso di ioni H^+, non solo imputabile ad una maggiore attività dell'enzima pmH^+-ATPasi ma probabilmente ad una funzione di sistemi H^+-ATPasi indipendenti o sistemi redox a livello di membrana.

Il secondo obiettivo ha rappresentato un primo approccio per esplorare gli effetti che interessano lo stato antiossidante della radice dei due genotipi di pomodoro, dopo brevi esposizioni a livelli tossici di tale elemento (0, 320 e 640 μM). In particolare, è stato valutato se l'accumulo di perossido d'idrogeno (H_2O_2) nei tessuti radicali, evento solitamente riscontrato come risposta all'eccesso di B in diverse specie vegetali, potesse essere associato con i livelli di malondialdeide (MDA), uno dei prodotti di accumulo di perossidazione lipidica delle membrane cellulari, e con l'induzione di meccanismi di scavenger mediati da enzimi antiossidanti quali l'enzima superossido dismutasi (SOD). Infine, è stata valutato l'attività dell'enzima perossidasi (POD) quale possibile responsabile del processo di lignificazione a livello radicale in risposta all'eccesso di B. I risultati ottenuti confermano che l'eccesso di B viene prontamente percepito a livello radicale, dove è in grado di causare un repentino e improvviso incremento dei livelli di H_2O_2 e un concomitante aumento dei prodotti di degradazione della membrana (MDA) nei tessuti radicali. Tale comportamento interesserebbe maggiormente l'ibrido Ikram, nel quale, infatti, a partire dall'ottava ora di esposizione a 320 μM B si riscontra un brusco e progressivo aumento dei livelli di H_2O_2 e MDA, mentre solo la concentrazione massima di B (640 μM) è in grado di provocare l'accumulo di tali prodotti nelle radici dell'ibrido Losna. I risultati ottenuti sembrerebbero confermare e giustificare in parte la diversa sensibilità dei due ibridi all'eccesso di B, come

riportato nel primo lavoro della presente tesi. Tenendo comunque presente la duplice natura di alcune specie reattive dell'ossigeno (ROS) e dei prodotti della perossidazione lipidica, resta da comprendere se il loro accumulo all'interno di radici esposte per breve tempo ad eccesso di B possa essere considerato semplicemente un inizio dello sviluppo di un danno ossidativo, e/o avere la funzione di allertare i sistemi di difesa antiossidante. A tal proposito, poiché l'attività svolta dalla SOD costituisce il primo sistema di difesa antiossidante contro i danni causati dai radicali derivanti dall'ossigeno, è stato eseguito un saggio di attività di tale enzima in radici di entrambe i genotipi. I risultati hanno mostrato il coinvolgimento di questo enzima in risposta a concentrazioni tossiche di B. In particolare, l'ibrido Ikram, oltre a manifestare un'attività insita maggiore della SOD rispetto a Losna, presenta anche un importante incremento di tale attività in presenza della più elevata concentrazione di B. Tuttavia, non è possibile stabilire un chiaro effetto dell'eccesso di B sull'attività della SOD nelle radici di entrambe i genotipi di pomodoro a causa della elevata variabilità riscontrata nel breve periodo considerato. È possibile comunque affermare che l'attività di tale enzima non rappresenti un fattore critico nel meccanismo di tolleranza alla tossicità da B. L'eccesso di B nel mezzo di crescita induce anche un sostanziale incremento dell'attività della POD, apparentemente corrispondente con l'accumulo del suo substrato ossidante, ovvero H_2O_2. Poiché tra i composti fenolici derivanti dai processi di catalisi operati dalla POD sono compresi alcuni precursori delle molecole di lignina, è possibile supporre che l'attività di tale enzima si concentri principalmente nelle radici incidendo sul processo di lignificazione dei tessuti radicali in risposta a eccesso di B. Tuttavia, nonostante l'attività della POD fosse superiore di circa il 40% in Ikram rispetto a Losna dopo 48 ore di trattamento, non sussistono differenze statisticamente significative che consentono di ipotizzare un differente comportamento di tale enzima nei due genotipi di pomodoro. Quindi, come precedentemente riportato per la SOD, nessun coinvolgimento specifico può essere attribuito all'attività della POD nello sviluppo di tolleranza al B.

Il terzo obiettivo è stato quello di valutare gli effetti dell'innesto erbaceo sulla crescita e la morfologia radicale di piantine di pomodoro allevate in condizioni di eccesso di B. L'interesse per il pomodoro innestato nasce dal fatto che questa tecnica è sempre più utilizzata in Italia e in molti altri Paesi come strumento di difesa nei confronti di numerosi agenti patogeni presenti nel terreno e per aumentare la resistenza alla salinità. Tale studio mirava principalmente ad accertare la plasticità morfologica sia dell'intero apparato radicale sia intra-radicale nel determinare la maggiore tolleranza allo stress da eccesso di B delle piante innestate rispetto a quelle non innestate. In particolare, il lavoro è stato eseguito su tre tipologie di piante di pomodoro: *Ungrafted* (ibrido Ikram non innestato), *Self-Grafted* (ibrido Ikram

innestato su Ikram) e *Grafted* (ibrido Ikram innestato su Arnold – portainnesto resistente alla salinità). Tali piante sono state sottoposte a trattamenti con 0 - 5 - 10 - 15 mg/L di B, per 0, 7, 14, 21 giorni. Su tale materiale vegetale è stata inizialmente valutata la crescita del germoglio, attraverso la misura del peso secco, ed il contenuto di clorofilla delle foglie; infine, è stata condotta un approfondita analisi della morfologia radicale. I risultati hanno permesso di evidenziare una maggiore tolleranza delle piante *grafted* all'eccesso di B, rispetto alle *self-grafted* e *ungrafted*. In particolare, sia il peso secco del germoglio sia il contenuto in clorofilla delle foglie delle piante *grafted* esposte a dosi eccessive di B subivano una riduzione inferiore rispetto le piante *self-grafted*, le quali mostravano comunque una minore diminuzione di tali parametri rispetto le *ungrafted*. La riduzione del peso secco del germoglio, così come del contenuto in clorofilla delle foglie, iniziava a manifestarsi dal 14° giorno di trattamento ed interessava maggiormente lo stelo rispetto le foglie di tutte le tipologie di piante considerate. Inoltre, da un'analisi della distribuzione spaziale del contenuto in clorofilla lungo i palchi del germoglio, le piante *grafted* continuavano a mostrare una risposta migliore ai differenti trattamenti di B poiché subivano una riduzione di tale parametro solo a livello della prima foglia, ovvero la foglia più vecchia presente nella pianta, generalmente considerata il primo bersaglio in condizioni di eccesso di B. Contrariamente, nelle piante *self-grafted* e *ungrafted* un contenuto inferiore in clorofilla era registrato anche nella quarta e nella quinta foglia, ovvero nelle foglie più giovani, lasciando ipotizzare che in presenza di eccesso di B tale elemento continuava ad essere assorbito e trasportato lungo il flusso traspiratorio raggiungendo e accumulandosi nelle foglie più giovani dove provocava il danno. Anche l'analisi della morfologia radicale confermava che le piante *grafted* presentavano una migliore risposta alle elevate concentrazioni di B, mostrando un apparato radicale più lungo e sottile rispetto le *self-grafted* e *ungrafted*. Inoltre, la lunghezza radicale era ridotta sensibilmente dalle elevate concentrazioni di B, in maniera sensibile nelle piante *self-grafted* ma ancora di più in quelle *ungrafted*. Tale diminuzione di lunghezza radicale è causata soprattutto dalla ridotta biomassa allocata nell'apparato radicale ma non da effetti sui parametri strutturali della radice, finezza e densità di tessuto radicale. Ancora più interessanti sono stati i risultati ottenuti attraverso l'analisi intra-radicale, nel quale sono state prese in considerazione le variazioni (in termini di lunghezza) delle differenti classi di diametro presenti nella radice. I risultati ottenuti mostrano un influenza dell'eccesso di B e della tipologia d'innesto sulla composizione delle differenti classi di diametro radicale. In particolare, le piante *grafted* oltre a mostrare una maggiore lunghezza per la classe di diametro *'very fine'* (0-0.5 mm) rispetto le *self-grafted* ed *ungrafted*, erano le uniche a non mostrare alcuna modifica di tale parametro al variare dei trattamenti di B utilizzati.

Diversamente la lunghezza radicale per le classi di diametro *'fine'* e *'large'* non era differente tra le tre tipologie di piante ma era modificata dalla presenza di boro, riducendosi sensibilmente. Quindi, è possibile sostenere che l'apparato radicale svolga un ruolo strategico nella tolleranza della pianta all'eccesso di B ed è possibile affermare che, almeno nel caso del pomodoro, l'uso di specifici portainnesti potrebbe costituire una strategia alternativa per superare le problematiche legate a tale stress.

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CHAPTER 1 GENERAL BACKGROUND: ECOPHYSIOLOGY OF BORON AND PLANT NUTRITION

1.1 BORON CHEMISTRY

Belonging to third group in the periodic table (Tariq and Mott, 2007), boron (B) is considered as a typical metalloid element such as silicon (Si), arsenic (As) and germanium (Ge) (Nable *et al.*, 1997), having properties intermediate between the metals and the non-metals (Argust, 1998). The small atom size (4,62 cm^3mol^{-1}) together with three valence electrons and a high ionization energy defined its unique and complex chemical properties (Greenwood and Earnshaw, 1984). Boron is electron-deficient, possessing a vacant *p*-orbital and it is normally found in B^{3+} state. However, B chemistry is of covalent stable B compounds and not of B^{3+} because of its very high ionization potentials. It is usually assigned a +3 valence because it combines with more electronegative elements. Furthermore, it has tendency to form anionic rather than cationic complexes.

Boron is not present on Earth in its elemental form, it occurs in nature in combination with oxygen as borates such as the borax ($Na_2B_4O_7 \bullet 10H_2O$), less frequently as boric acid [$B(OH)_3$ or H_3BO_3] and very rarely associated with fluorine, as anion BF^-_4 (Power *et al.*, 1997). On the contrary, in plant and animal cells, at pH about 7.5, in the citoplasmatic compartment more than 99.95% of the B exists in the H_3BO_3 form while the remaining part is present as borate [$B(OH)^-_4$].

Boron occurs in aqueous solution as H_3BO_3, hydrolyzing reversibly to the borate ion [$B(HO)^-_4$] according to the reaction by Baes and Menders (1976):

$$B(OH)_3 + H_2O = B(OH)_4 + H^+ \longleftrightarrow pK_a = 9.2$$

In accordance with electronic B configuration, at a pH < 7, H_3BO_3 acts as a very weak Lewis acid, and its activity seems to be related to the acceptance of OH^- rather than the donation of H^+. Therefore, in neutral or slightly acid soils, B is located mainly in the form of un-dissociated boric acid (Raven, 1980).

Both boric acid and borate can quickly react with many different types of molecules (Brown *et al.*, 2002). In particular, they are able to form esters and complexes with a wide variety of mono- di- and poly-hydroxy compounds. The boric acid binds to a lot of sugars containing a furanoid rather than a piranoid ring as ribose, the main constituent of the RNA, (Loomis and Durst, 1992; Goldbach, 1997) and to apiose, mannitol, mannan and polymannuronic acid, essential constituents in plant cell wall. O'Neill *et al.* (2001, 2004) demonstrated that borate forms a cross-link

with apiose residues of rhamnogalacturonans II (RG-II), important components of plant cell wall, which is essential for normal leaf expansion in *Arabidopsis thaliana*. Although, Loomis and Durst (1992) sustained that the B complexes formation with ribonucleotides was a probable cause of boron toxicity, recently the ability of B to stabilize ribose and to form borate ester nucleotides, makes it a "prebiotic element" which provides an essential contribution to the "pre-RNA world" (Scorei, 2012; Grew *et al*., 2011).

Furthermore, the boric acid has a greater affinity to the organic *cis*-diols (Boeseken, 1949), and consequently some *o*-diphenols such as caffeic and hydroxiferulic acids, important precursors for the lignin biosynthesis in dicots, are able to form stable borates (McClure, 1976).

Both these B complexes with sugars and organic *cis*-diols could be considered fundamental in the living world. Indeed, the stabilization of these molecules could represent a most probable defense mechanism of genetic material, providing its thermal and chemical stability in hostile environments, throughout the evolution of life (Scorei, 2012; Grew *et al*., 2011).

In addition, the ubiquitous presence of OH groups within biological molecules allows the formation/dissociation of many B complexes (Power and Wood, 1997) which generally occur spontaneously through rapid kinetics mainly influenced by pH (Woods, 1996). Furthermore, the stability depends on the nature of the constitutive molecular groups of B complexes. For example, the presence of nitrate would increase the steadiness of B complexes since it is able to bond hydrogen molecules which confer greater electrostatic stability to them (Woods, 1996). The same behavior was observed with the coenzyme NAD^+ which shows a greater ability to form more stable borates complexes than its reduced form NADH (Brown *et al*., 2002). This could indirectly affect some enzymatic activities depending on NAD^+ coenzyme causing significant metabolic disorders (Wimmer *et al*., 2003).

1.2 BORON SOURCE AND DISTRIBUTION

Boron is widely distributed in nature and its major reservoirs in the biosphere are shown in Table 1 (Kot, 2008). Its turnover and the extent of flows through which B moves among the different environmental compartments seem to be less clear. The B inorganic forms, usually found in water, soil, and atmosphere, originate from both natural and anthropogenic sources. Boron occurs mainly in silicate minerals at approximately 10 mg kg^{-1} concentration in the Earth's crust. It can be found as borosilicates in igneous, metamorphic and sedimentary rocks and its distribution among rock classes and types proposed by Krauskopf

(1972) has reported in Table 1. Natural weathering of sedimentary rocks is thought to be the primary source of boron compounds in water and soil (Butterwick *et al.*, 1989) while it is predominantly released to the atmosphere from oceans (65-85%), volcanoes, and geothermal steam (Graedel, 1978). Global releases of elemental boron through these processes are estimated at approximately 360,000 metric tons annually (Moore, 1991). B-rich deposits around the globe are located in California, Australia, China, Russia, Argentina, although Turkey is ranked top with its share of almost 73% in the global boron reserve. In particular, the western Turkey contains 60% of B deposits causing a significant problem for agriculture and water resources (Ozkurt, 2000).

The B level in atmosphere averages ~ 20 ng m^{-3} with a range of 0.5 - 80 ng m^{-3}. Because borates exhibit low volatility, B would not be expected to be present to a significant degree as a vapor in the atmosphere. Atmospheric emissions of borates and boric acid in a particulate (<1–45 μm in size) or vapor form occur as a result of volatilization of boric acid from the sea, volcanic activity, mining operations, glass and ceramic manufacturing, the application of agricultural chemicals, and coal-fired power plants.

Rock class	Rock type	Boron (mg Kg^{-1})	Mineral class	Mineral type	
Igneous	*Granite*	*15*	***Hydrous borates***	*Borax*	$Na_2B_4O_7{\cdot}10\ H_2O$
	Basalt	*5*		*Kernite*	$Na_2B_4O_7{\cdot}4\ H_2O$
				Colemanite	$Ca_2B_6O_{11}{\cdot}5\ H_2O$
Sedimentary	*Limestone*	*20*		*Ulexite*	$NaCaB_5O_9{\cdot}8\ H_2O$
	Sandstone	*35*			
	Shale	*100*	***Anhydrous borate***	*Ludwigite*	Mg_2FeBO_5
	Soils	*7-80*		*Kotoite*	$Mg_3(BO_3)_2$
			Complex borosilicates	*Tourmaline*	$Na(Mg,Fe,Mn,Li,Al)_3Al_6Si_6O_{18}(BO_3)_3(OH,F)_4$
				Axinite	$(Ca,Mn,Fe)_3Al_2BO_3Si_4O_{12}(OH)$

Table 1 *Boron major reservoirs in the biosphere*

Boron is found in soils, from 10 to 300 mg kg^{-1}, typically ranging from 0.1 to 0.5 mg L^{-1} (Howe, 1998) and in plants (10% of the total soil B content), but both these issues will be deeply discussed in the following chapter sections.

In seawater, the concentration of boron ranges from 0.5 to 9.6 mg kg^{-1} with an average of 4.6 mg kg^{-1} (Power and Woods, 1997). High levels of boron are also present in domestic wastewater (0.5-2 mg L^{-1}) and in groundwater (0.3-100 mg L^{-1};

e.g. in Mediterranean countries, approximately 8 mg L^{-1}). These values can significantly increase near areas with geothermal activity because of its high concentration in thermal waters (0.2 to 72 mg L^{-1}). For example, the B concentration in water supplies can reach values of 3-13 mg L^{-1} in exposed areas of Cornia Valley (Italy) (EU research project BOREMED, http://boremed.brgm.fr/). This project showed that the presence of B in groundwater contamination is not caused by human activity due to urban agricultural and/or industrial residues (release of detergents, fertilizers, etc.), but by the Pleistocene geothermal area.

The B level in fresh water surface ranged from 0.001 to 2 mg L^{-1} in Europe (mean value 0.6 mg L^{-1}), Pakistan, Russia, reaching instead highest B values (4-26) mg L^{-1} in South America. Consistent with B values observed in ground- and surface-waters, B concentrations are also found in drinking-water (0.01 - 15 mg L^{-1}) due to the leaching from surrounding geology and wastewater discharges and the difficulty to remove it by conventional drinking-water treatment methods. Finally, boron is also present in irrigation water where it causes a serious problem for domestic and agriculture utilizations (Polat *et al.*, 2004), affecting at B level higher than 1 mg L^{-1} yield of sensitive crops. Several examples have been reported in in Spain (Salinas *et al.*., 1981), Arizona (Ryan *et al.*, 1977), northern Greece (Sotiropoulos, 1997) and Philippines (Dobermann and Fairhurst, 2000).

Boron is also found in foods and vegetables. In human health, diets rich in fruits, vegetables, legumes and nuts may provide the B amount needed to guarantee the function or composition of several body systems, including the brain, the skeleton and the immune system (Nielsen, 2008). An intake of over 1 mg day^{-1} is desirable but probably not more than 13 mg day^{-1} (Nielsen, 1997). Neurological effects, weight loss, testicular atrophy and skeleton malformation have been reported in animals with excessive boron intake (Yazbeck *et al.*, 2005). It is an essential element also for diatoms, cyanobacteria, other marine algal flagellate (Loomis and Durst, 1992) and animal species. Although, the B function in animals is not completely understood (Devirian and Volpe, 2003).

Anthropogenic sources of boron are considered to contribute a lesser amount to the environment (7-18%) than natural processes. They include releases to atmosphere from power and chemical plants, and manufacturing facilities (Nable *et al.*, 1997). On the contrary, fertilizers, herbicides, and industrial wastes are among the soil contamination sources. The occurrence of B contamination in water can come directly from industrial wastewater and municipal sewage, as well as indirectly from air deposition and soil runoff. For example, coal fly ash represents an important B source in both soil and water environments being easily leached from coal ash nearby coal-ash wastes dump (Wood and Nicholson, 1998) or coal-fired power station

(Hermann, 1994). Further, municipal and wastewater used for irrigation may contribute to high boron concentrations in agriculture systems (Tsadilis, 1997). Borates in detergents, soaps and personal care products, in pharmaceuticals (as pH buffers), can also contribute to the presence of boron in water (WHO, 1998).

1.3 BORON IN SOIL

Boron occurs in soils as H_3BO_3 and partially as $B(HO)_4^-$ distributed unevenly in the circulating solution and in the organic and mineral fractions. In particular, the un-dissociated form H_3BO_3 prevails in the soil solution, and only at pH > 9.2, the $H_2BO_3^-$ form becomes predominant. In soils, B is considered the more mobile element and often deficient among all the trace elements. In a study conducted by FAO on the micronutrients status in soils, the B deficiency was the most common micronutrient problem, affecting at least 8 million hectares worldwide (Sillanpaa, 1982; Tariq and Mott, 2007). Boron deficiency is found primarily in humid regions with well-drained soils or in sand soils as reported in some regions of China, Japan and USA (Tanaka and Fujiwara, 2008). High rainfall of these countries together with high boron solubility in soil solution may be the major reasons of B deficiency (Shorrocks, 1997). However, at slightly high concentrations, B may become toxic for plants as a range between B deficiency and toxicity is relatively narrow (Gupta, 1993), making difficult the B management in plant-soil system. Both B deficiency and toxicity are associated with plant disorders and reduction of crop yield and quality. Soils excessively B fertilized, irrigated with sewage debris or salt water may contain toxic B concentrations. Boron toxicity generally occurs when soils contain greater than 12 mg B Kg^{-1} (Hall, 2010) and it is usually confined to areas with less than 550 mm annual rainfall. Low rainfall in dry regions also means little soluble boron is leached from the root zone and soil profile (McDonald *et al.,* 2010).The amount of water required to leach boron is approximately three times that required for sodium chloride leaching (Moore, 2004). The B toxic soils occur in Australia (Western Australia, South Australia and Victoria), Jordan, Malaysia, Peru, Chile (North), India, Israel, Mediterranean areas (Turkey, Morocco), and USA (California), (Nable *et al.*, 1997; Brennan and Adcock 2004; Kot, 2008; Tanaka and Fujiwara, 2008).

Based on climatic zones, the concentrations and the chemical pool of B in soils vary. The boreal, tropical and temperate regions have low B concentrations ranging from 1 to 2 $\mu g\ g^{-1}$. Conversely, the soils of arid and semiarid regions contain rather high B concentrations between 10-40 $\mu g\ g^{-1}$ (Evans and Sparks, 1983).

The main sources of B in most soils are B-containing primary minerals such as tourmaline [Na (Al, Fe, Li, Mg, $Mn)_3Al_6\ Si_6O_{18}\ (BO_3)_3$ (OH, $F)_4$] and the volatile

emanations of volcanoes (Chesworth, 1991). Other more common B-containing minerals are: the ulexite, Na $Ca[B_5O_6(OH)_6]*5H_2O$; the borax, Na_2 $[B_4O_5$ $(OH)_4]*8H_2O$; the colemanite, $Ca_2[B_3O_4$ $(OH)_3]*2H_2O$, a less soluble mineral, and the kernite, $Na_2[B_4O_5(OH)_4]*2H_2O$, a less hydrated borax. Because of the limited solubility of such B-containing minerals in soils and their resistance to weathering, B is not readily available to plants (Nable, 1997; Zerrari *et al.*, 1999). The B adsorbed on the surfaces of the colloidal soils does not affect the amount of B responsible of toxicity to plants (Ryan *et al.*, 1977; Keren *et al.*, 1985a; Keren *et al.*, 1985b). Groundwater, for the addition of artificial residues mining processes, fertilizers or residues of fossil fuels (Nable, 1997), contributes to raise the B level in soils. However, the major source of B in soils is probably the irrigation water (Chauhan and Power, 1978; Keles *et al.*, 2004). The threshold B concentration in water irrigation has been established for sensitive (0.3 mg L^{-1}) and tolerant (2 mg L^{-1}) crops, taking into account the physical-chemical properties of the soil and the B soil interaction (Keren, 1996).

Total, acid soluble and water soluble are the three B pools present in soils. The total B content has little bearing on the status of available B to plants which results to be about 10% of the total content of B in the soils (Power *et al.*, 1997). The water soluble B content frequently ranged from 7 to 80 μg g^{-1} in soils (Krauskopf, 1972) providing a general indication of B supply to plants. In relation to water-soluble B concentrations in soils, Fleming (1980) defines three categories: insufficient (<1μg mL^{-1}), sufficient (1-5μg mL^{-1}) and toxic (> 5μg mL^{-1}) boron for normal plant development. This classification has been later reviewed by Sillanpaa (1982) and Shorrocks (1993) with some modifications which show however that water soluble B in soils > 0.5 μg g^{-1} is sufficient for plant growth of many crops. However, water soluble boron depends on soil system, crop species, lime application and irrigation management and environmental conditions (Tariq and Mott, 2007).

Boron sorption-desorption processes regulate the water-soluble B availability acting as source-sink for plant uptake in soils solution which in turn is influenced by soil physical-chemical properties (Keren and Bingham, 1985; Chen *et al.*, 2002; Arora and Chahal, 2005). Among these, the soil pH has been reported as the main factor affecting the B adsorption in agricultural soils (Keren and Bingham, 1985; Saltali *et al.*, 2005; Soares *et al.*, 2008), together with soil texture, soil moisture, clay content, Al and Fe (hydr)oxides, clay minerals, calcium carbonate and organic matter (Goldberg, 1997; Arora *et al.*, 2002; Goldberg *et al.*, 2005; Arora and Chahal, 2007; Goldberg *et al.*, 2008; Shafiq *et al.*, 2008; Arora and Chahal, 2010).

A positive correlation between B adsorption on clay minerals, hydroxyl- Al and the increase of pH values in soils has been reported (Gupta, 1993; Keren, 1996;

Goldberg, 1997). Indeed, at pH below 7.0, the predominant form H_3BO_3 shows a relatively low affinity to the clay, while, in alkaline pH range, $B(HO)^-_4$ species increased rapidly reaching the maximum of adsorption around pH 9.0 (Bingham *et al.*, 1971; Elrashidi and O'Connor, 1982). Different empirical models have been applied to describe adsorption reactions such as Langmuir and Freundlich adsorption isotherm equations (Goldberg, 2003). Recently, Steiner and Lana (2013), analyzing some soils of Paraná (Brazil) confirmed that B adsorption was dependent on soil pH, increasing as a function of pH, but was also affected by soil properties such as the organic matter, clay and aluminum oxide content.

Excessive moisture in the soil can cause significant loss of B related to the phenomena of leaching (Kot, 2008), while in clay soils the B is more easily retained partly because of the strong capacity of the clays to form stable complexes with this microelement (Mattigod *et al.*, 1985). On weight basis, illite is the most reactive among the common clay minerals whereas kaolinite is the least reactive (Keren, 1996). The presence of organic matter in the soil provides an important reserve of B due to the presence of this element in many organic compounds. Boron can be absorbed on organic matter and sesquioxides by ligand exchange mechanism (Yermiyahu *et al.*, 1988). Some authors have shown not only that the presence of B in the soil organic matter can be higher than that in the mineral fraction (Yermiyahu *et al.*, 2001; Lemarchand *et al.*, 2005), but also that the absorption of B is positively influenced through fertilization with organic materials (Yermiyahu *et al.*, 2001). Coarse textured soils often contain less boron than fine textured soils (Sarkar *et al.*, 2008).

In arid or semiarid areas, B toxicity is frequently associated with salt stress (Goldberg, 1997) as observed in the Lluta valley (Northern Chile) and in the San Joaquin Valley (California) (Bastías *et al.*, 2004; Wimmer *et al.*, 2003). Interactive effects on stress responses have been clearly established, but the results are often contrasting indicating antagonistic or synergistic interactions even within the same plant species (Mittler, 2006; Yermiyahu *et al.*, 2008). Bingham *et al.* (1987) found that plant response to boron was independent of salinity levels in the soil. On the other hand, salinity seemed to alleviate B toxicity decreasing total shoot B concentrations (Alpaslan and Gunes, 2001; Ismail, 2003; Diaz and Grattan, 2009). Wimmer *et al.* (2003) found that salinity can aggravate boron toxicity symptoms in several plant species because of combined stresses significantly increased soluble boron concentrations at intra and intercellular level. So far, conclusive considerations on mutual relationship between salt stress and B toxicity are lacking yet (Yermiyahu *et al.*, 2008; Grieve *et al.*, 2010).

1.4 BORON IN PLANTS

The presence of B in plants was reported for the first time in 1910, but only later Katherine Warington (1923) claimed the importance of B for growth and development in broad beans and other legumes. In the same period, the B requirement for six non-leguminous dicots and one graminaceous plant was also demonstrated (Sommer and Lipman, 1926). Currently, based on the B requirement for their growth and development, plants can be divided into four classes: i) lactifers (latex-forming species); ii) legumes; and subsequently iii) the remaining dicots and rather all monocots families leaving out; and iv) graminacous plants, considered to be the less-demanding (Bonilla *et al.*, 2009). Excluding lactifers, the higher B requirement in all other plants was presumably due to a higher content of *cis*-diols configuration compounds within the cell wall, such as pectins and polygalatturans (Loomis and Durst, 1992). This hypothesis was based on diverse content of molecules capable of creating B complexes in the cell wall radicals which was equal to 3-5 g g^{-1} dry weight in grasses increasing up to 30 g g^{-1} in dicotyledonous species (Tanaka *et al.*, 1967). Therefore, such differences could support the diversity in the B-requirement among plant species for reaching an optimal growth (Marschner, 1995).

By now, B is considered an essential micro-nutrient for normal plant growth (Emebiri *et al.*, 2009), unevenly distributed within plants and especially found in tissues of reproductive structures (Saleem *et al.*, 2011). Crop species and cultivars show varying ranges at which B is considered adequate. For example, in monocots, B concentrations range from 1 to 6 mg kg^{-1} while in most dicots from 20 to 70 mg kg^{-1}. However, the required B range necessary for optimal plant growth is very narrow (Moore, 2004; Bingham *et al.,* 1987; Grieve and Poss, 2000). For example, in rice B concentration between 6-15 mg kg^{-1} is considered adequate while just below and above these values, B becomes deficient or toxic, respectively (Dobberman and Fairhurst, 2000). Both deficient and toxic B levels caused plant disorders reducing the yield and quality of final products.

Boron is involved in many important processes in higher plants such as: i) the transport of sugars and carbohydrate metabolism; ii) the cell wall synthesis and the lignification process; iii) the maintenance of the integrity of the plasma membrane and of its function; iv) the stimulation of the nucleic acids metabolism; v) the indoleacetic acid metabolism; vi) the ascorbate/glutathione cycle; vii) the phenolic compounds metabolism; vii) the pollen tube formation; viii) the nitrogen metabolism; ix) the photosynthesis and also x) several enzymatic activities (Figure 1) (Paull, 1990; Moore, 2004; Rehman *et al.* 2006; Reid, 2010).

However, its primary role, widely recognized, is in the cell wall and the plasma membrane where it is required as structural component conferring stability (as already reported by Warington, 1923).

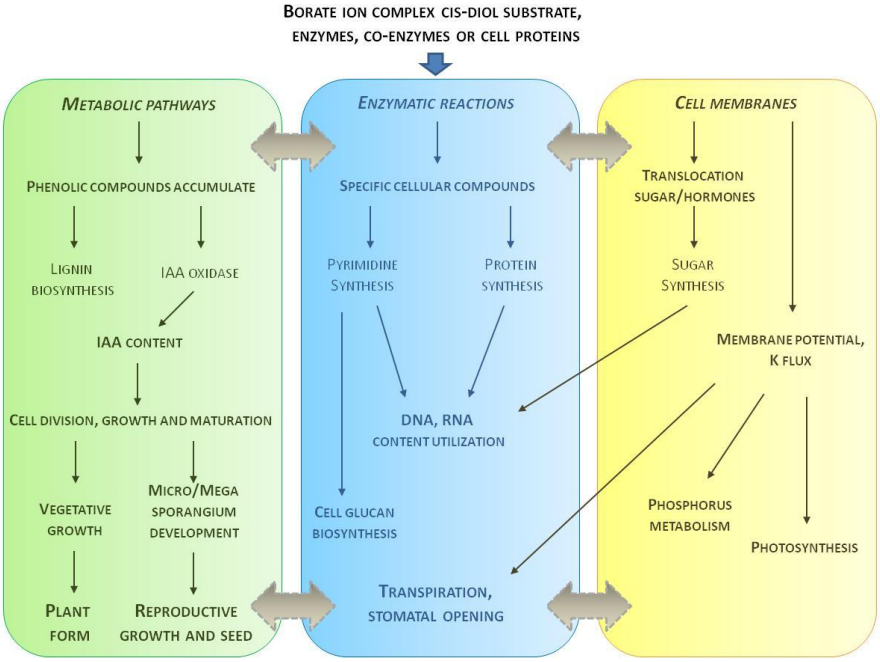


Figure 1. *Boron targets*

1.4.1 BORON IN THE CELL WALL AND MEMBRANES

Ninety percent of B content in plant cells is localized in the cell wall fraction (Blewins and Lukaszewski, 1998; Loomis and Durst, 1992; Hu and Brown, 1994). Such evidence highlighted strong influence of B in the organization of cells wall proteins, pectins and/or precursors during plant growth and development (Torosell, 1956; Spurr, 1957). Indeed, in B deficient plants, the structural abnormalities in the composition of cell wall and middle lamella (Matoh *et al.*, 1992) caused a growth block on the apical meristems of root and stem (Brown *et al.*, 2002), on the pollen tubes (Schmucker, 1933), as well as the fragility in developing leaves and petioles (Loomis and Durst, 1992; Shorrocks, 1997; Goldbach, 1997).

The B functional role in cell wall organization has been firstly demonstrated after the isolation of a B-polysaccharide complex from radish root cell walls (Matoh *et al.*,1993) on which a rhamnogalacturonan II (RGII) polysaccharide was later characterized (Kobayashi *et al.*, 1996; O 'Neil *et al.*, 1996). In particular, RGII was

cross-linked by 1:2 borate- diol diester to form the dimeric RGII via cis-diol groups of two apiose residues forming a stable three-dimensional network (O' Neil *et al.*, 1996). In the same period, the presence of RGII-B complex in cell wall of other 22 species has been demonstrated (Matoh *et al.*, 1996). In absence of B, Fleischer *et al.* (1999) observed a wider formation of cell wall holes than normal inside the three-dimensional structure' due to a lack of dB-RGII, suggesting a new B functional role in cell wall. The presence of improper pore, in B deficient cells, may affect physiological important processes such as the incorporation and transport of polymers into the wall. In this respect, Dannel *et al.* (2002) stated that the B- RGII complexes contributed to the porosity and strength of the cell wall. Moreover, O' Neil *et al.* (2001, 2004), using the mur1 mutant in Arabidopsis thaliana characterized by abnormal sugar composition of RGII, defined at molecular basis the importance of B-RGII for the normal leaf expansion. Hence, the B requirement was strongly associated to the RGII content of the cell walls in different plant species (Reid *et al.*, 2004) and a reduced production of pectic substances or precursors of the cell wall in plants exposed to B deficiency has been reported (Bonilla *et al.*, 2009), although there is no convincing evidence to suggesting a direct involvement of B in the process of cell wall synthesis.

Boron effects on organisms lacking in cell walls underlined the B essentiality in plant growth and development beyond its role in cell wall structure (Bennett *et al.*, 1999; Läuchli, 2002). Many authors have speculated that B plays a structural role inside the plasma membrane which may explain the large number of B effects on it. Cakmak *et al.* (1995) sustained that B stabilizes the plasma membrane structure by forming complexes with its components. They observed that the loss of potassium, glucose, phenols and amino acids in sunflower leaves subjected to shortage of B underlined the B role in the integrity of plasma membranes. Several studies have shown that B affects the structure and function of membrane and especially of plasma membrane (Blevin and Lukaszweski, 1998). A B adequate supply in plants triggered a multitude of events including the membrane hyperpolarization of cell radicals (Schon *et al.*, 1990), the stimulation of ferricyanide-dependent H^+ release (Goldbach *et al.*, 1990) and of H^+-ATPase and NADH oxidase activities (Barr et al., 1993) and finally of ion uptake (Blevin and Lukaszweski, 1998). On the contrary, B deficiency reduced rubidium (Rb^+) and phosphorus (P) uptake in Vicia faba, sunflower and maize roots which was restored after B addition (Robertson and Loghman, 1974; Goldbach, 1984). Boron deficiency and toxicity also inhibited ATP-dependent H^+ pumping and vanadate-sensitive ATPase activity (Pollard *et al.,* 1977; Ferrol et al., 1993). In cell suspension of carrot and tomato cells, B reduced the ferricyanide-induced proton release mediated by vanadate suppression, suggesting

the involvement of plasma membrane proton pump in this process (Golgbach *et al.*, 1990). Therefore, B not only stabilized the membrane-molecules with cis-diol groups (Bolanos *et al.*, 2004) but also regulated its function. Recently, in tobacco plants boron deficiency caused a nitrate content decrease due to the lower net nitrate uptake rate as a consequence of root plasma membrane H^+-ATPase (PMA2) transcript reductions (Camacho-Cristobal *et al.*, 2007, 2008).

Moreover, it has been hypothesized that B may be involved in the structure of so-called "membrane rafts" particularly "lipid rafts", physiologically active membrane fractions with relevant functions in signal transduction and useful as binding sites for glucosilfosfatil-inositol (GPI) proteins (Brown *et al.*, 2002). They are characterized by high concentrations of glycolipids and glycoproteins, providing a significant number of B complexing sites. In addition, these fractions also contain either sugars such as galactose, mannose or amino acids such as serine and tyrosine able to link with the B. For this reason, B seemed to play a specific function in membrane stability, integrity and function of membrane rafts. Recently, Voxeur and Fry (2014) characterized, in rose cell cultures, a glycosylinositol phosphorylceramides (GIPCs), the major sphingolipids in lipid rafts able to form a GIPC-B-RGII complex (Borner *et al.*, 2005),. using a thin-layer chromatography (TLC) and mass spectrometry (MS) approach. They concluded that: i) B played a structural role in plasma membrane; ii) high B level disrupting the membrane components was responsible for membrane phytotoxic effect; iii) GIPCs facilitatedvored B-dependent RGII dimerization process; and finally iv) GIPC-B-RGII gave for the first time, the molecular explanation of the wall-membrane attachment sites (Voxeur and Fry, 2014).

1.4.2 BORON AND PLASMA MEMBRANE H^+-ATPASE INTERACTION

The plasma membrane (pm)H^+-ATPase is an important functional protein which plays a central role in plant physiology. The pmH^+-ATPase is involved in ATP hydrolysis to transport protons out of the cytosol into the apoplast establishing an electrochemical gradient across the plasma membrane (Duby and Boutry 2009). This gradient generates a proton-motive force which drives the secondary ion transport (Briskin and Hanson 1992; Morsomme and Boutry 2000; Palmgren 2001) such as nitrate (Santi *et al.*, 1995; 2003; Sorgonà *et al.*, 2010; 2011), phosphorus (Yan *et al.*, 2002), potassium (Schachtman and Schroeder 1994), and iron (Schmidt 2003; Dell'Orto *et al.*, 2000). In this way, this enzyme controls root nutrient uptake and xylem or phloem loading. Moreover, pmH^+-ATPase is involved in other important physiological processes, such as stomata opening, expansion growth, and cytosolic pH regulation. According to the acid-growth theory, auxin activates H^+-ATPase that

extrudes protons which in turn decreasing the apoplastic pH activates enzymes involved in cell-wall loosening (Hager 2003). A higher concentration of H^+ in the apoplast may also activate cell-wall proteins such as expansins (Cosgrove 2000) contributing to increase the cell-wall extensibility by breaking the load bearing bonds (Keller and Cosgrove 1995; Purugganan *et al*., 1997).

The pmH^+-ATPase is encoded by a multigene family showing several isoforms of which 9-12 have been already identified in different plant species. Several isoforms related to nutrient transport and cell growth are widely expressed in most plant tissues (Arango *et al*., 2003; Gaxiola *et al*., 2007). They may have different features, such as substrate affinity, *Vmax,* and pumping efficiency (Luo *et al*., 1999). The pmH^+-ATPase activity is controlled by an auto-inhibitory domain at the C-terminus (Palmgren *et al*,. 1991) whose modifications can change the pumping efficiency of the enzyme (H^+ transport /ATP coupling).

Several reports demonstrated that the presence of B in the root medium increased plant growth. Since B is mainly localized in the cell wall (Hu and Brown, 1994; Hu *et al*,. 1996) and cross-linked with rhamnogalacturonan II (O'Neill *et al*., 2004), it can be considered to be an important factor in cell wall extensibility and plant growth stimulation (Hu and Brown 1994; Findeklee and Goldbach 1996). The B-stimulated activity of plasma membrane NADH oxidase and H^+ secretion has been reported in cultured carrot cells (Barr and Crane., 1993). In sunflower root cells and leaved elodea (*Elodea densa*) leaf cells, a significant membrane depolarization after cells movement from B containing to B-free solution was observed, confirming the micronutrient effects on proton secretion and electrical potential gradient generation across the membrane (Blaser-Grill *et al*., 1989). It has been assumed that the B-induced stimulation of plant growth is caused by changes in pmH^+-ATPase activity (O'Neill *et al*., 2004).

Further, the pmH^+-ATPase activity is a crucial factor in the plant survival under a variety of environmental stresses, such as salt (Vitart *et al*., 2001) and aluminum (Ahn *et al*., 2001) treatments. Thus, it is reasonable to hypothesize that the root pmH^+-ATPase could be involved in the B stress adaptation. However, the evidence for its possible involvement under B excess is still lacking.

1.4.3 BORON AS SIGNAL

Although signal transduction pathways and plant sensing for mineral deficiencies are well known for macronutrients (Schachtman and Shin, 2007), the knowledge of most of micronutrients, especially B, is more limited. None of the proposed hypothesis fully explains how so many decisive pathways for plant development respond in short-term to B deficiency.

The first evidence of B as signal molecule required for quorum sensing was shown in bacteria (Chen *et al.* 2002), suggesting a similar role for B in both animals and plants. Although many studies explained how the variation in B-concentrations inside plant cells could trigger a cascade of signals which in turn altered the membrane-bound proteins conformation (reviewed by Goldbach and Wimmer, 2007), the role of B as signal molecule has not been clearly demonstrated yet.

This hypothesis could be supported by the rapid increase of proteins like actin and tubulin within the membranes of plant root cells of *Arabidopsis* and maize under B-deprivation, resulting in alterations in the pattern of polymerization, and consequently in the cytoskeleton assembly (Yu *et al.*, 2001, Yu *et al.*, 2003). Interestingly, B-deficiency could induce the expression of genes in response to stress such as *NIP5;1*, a member of the major intrinsic protein family, that encodes an essential protein for B absorption when B availability is limited for plant growth (Takano *et al.*, 2006). In this respect, a rapid signal movement from the cell wall to the cytoplasm that triggered the induction of the NIP5;1 or BOR family genes under B-deprivation was already hypothesized (Kobayashi *et al.*, 2004). The possible role of B as molecule signal was suggested because of its direct or indirect interaction with transcription factors (TF) (González-Fontes *et al.*, 2008). According to the target gene and the TF types (activators or repressors), this complex could regulate the expression of several genes, explaining either the diverse B-effects on so many physiological processes, and how a negligible amount of B into the protoplast can be decisive for the normal plant development (González-Fontes *et al.*, 2008). For example, several transcription factor genes belonging to MYB, WRKY and bZIP families were up-and down- regulated in response to short B deprivation (González-Fontes *et al.*, 2013).

The possible role of B as signal molecule in combination with Ca^{2+} has been observed in plants, animals and humans (Bolaños *et al.*, 2004; Gonzalez-Fontes *et al.*, 2014). Under B deficiency, Ca^{2+} addition reduced the negative effect on nodulation of N_2-fixing legume–rhizobia symbiosis and on the expression of some nodulation genes in *Medicago truncatula*, without reverting the abnormal cell wall structure in nodules (El-Hamdaoui *et al.*, 2003; Redondo-Nieto *et al.*, 2003). It has also been suggested that B deficiency caused an oxidative damage due to a reactive oxygen species (ROS) accumulation in the Arabidopsis root elongation zone similar to that observed under Ca^{2+} deficiency (Oiwa *et al.*, 2013). Considering that B and Ca^{2+} shared a key role in stabilizing cell wall structures (Goldbach *et al.*, 2001) and both up- and down-regulated the genes expression involved in several plant processes (Camacho-Cristóbal *et al.*, 2011), it was possible to suppose that B and Ca^{2+} could interplay in signaling events under B-deficiency in plants (Gonzalez-Fontes *et al.*,

2008; 2014). Recently, an intermediary role for Ca^{2+} and Ca^{2+}-related proteins in the transduction pathway triggered by B-deprivation has been proposed. In particular, cyclic nucleotides (cAMP or cGMP) appeared to be involved, as observed in different stress (Ma *et al.*, 2011), playing a major role to stimulate cyclic nucleotide-gated ion channels which allow Ca^{2+} to entrer the cytosol. Afterwards, the increase of Ca^{2} could trigger many physiological responses in plants (Gonzalez-Fontes *et al.*, 2014).

1.4.4 BORON UPTAKE BY ROOTS: PASSIVE AND ACTIVE MECHANISMS

Boron is taken up by plant roots from the soil solution as uncharged boric acid (Marschner, 1995), being an exception among all other plant mineral nutrients generally absorbed by roots in ionic form (Miwa and Fujiwara, 2010). The boric acid uptake mechanism in higher plants has been controversial for over 30 years but now the evidences support either passive or active processes (Dannel *et al.* 2000, 2002; Brown *et al.*, 2002; Tanaka and Fujiwara, 2008). Based on the high permeability of boric acid across lipid bilayers of biological membranes, boric acid uptake has been considered for a long time to be a passive process (Raven, 1980). The author firstly postulated the boric acid passive diffusion based on a theoretical lipid permeability coefficient of $B(OH)_3$ (8×10^{-6} cm s^{-1}) also claiming that active transport to maintain boric acid distribution across a membrane away a thermodynamic equilibrium was likely to be energetically expensive. Recently, the higher permeability of membranes to boron than other solutes were also observed in giant algal cells (Reid, 2014).

Several studies on boric acid absorption have been reported with contrasting results. Bingham *et al.* (1970) in excised barley roots demonstrated that boric acid absorption was a physical process. In contrast, Wilders and Neales (1971), using slides carrot disks and red been roots, sustained that B absorption consisted of two components, passive diffusion and active process. This hypothesis was also supported by Bowen and Nissen (1977) on barley seedlings. Afterwards, Brown and Hu (1994), studying cultured tobacco cells and sunflower roots, defined the boric acid absorption as a non-metabolic process. Thus, up to 1990, the general opinion was that, under normal or excessive B supply, the B absorption rate by roots was influenced by B concentration of the external solution, the formation of B complex within the cell wall and plant water flux (Hu and Brown, 1997). This idea was closely associable with the patterns of B deficiency and toxicity symptoms (Marschner, 1995).

Later, through direct measurements of B membrane permeability (Pf_B), Dordas and Brown (2000) confirmed the theoretical values indicated by Raven (1980) in artificial liposomes, but not in plasma membrane. Indeed, in isolated membranes of squash roots, the Pf_B values were slower either than the theoretical prediction of

Raven (1980) or artificial liposomes (Dordas *et al.*, 2000). Similar low values were found in individual charophyte alga cells (Stangoulis *et al.*, 2001). This discrepancy between calculated and experimental Pf_B was attributable to different lipid membrane composition which defined the properties and permeability of membranes. This hypothesis was also supported by results obtained with mutant lines of *Arabidopsis thaliana* differing in membrane lipid composition (Dordas and Brown, 2000). Furthermore, Dordas *et al.* (2000), using mercuric chloride and phloretin, two channel blockers, observed a partial inhibition (30-39 %) of boric acid permeation across plasma membrane vesicles of squash roots which was restored by 2-mercaptoethanol. From these results, they firstly provided the evidence that B entered into plant cells in part by passive diffusion through the plasma membrane lipid bilayer and in part through channels mediated transport (Dordas *et al.*, 2000). In addition, they firstly supported the hypothesis that boron can be taken up through facilitated diffusion via a MIPs superfamily, as discussed below (Dordas *et al.*, 2000). Brown *et al.* (2002) pointed out that acid boric passive permeation would be adequate to provide the B requirement for both canola and tobacco under adequate B supply (10 μM B), but not under limited B supply (1 μM B). Therefore, they sustained the role of membrane proteins in the facilitation of B transmembrane movement without precluding the existence, at low B levels, of an active B transport mechanisms needed to satisfy B plant requirement (Brown *et al.*, 2002).

A major shortcoming of the passive uptake hypothesis was that it could not explain either the observed differences in boric acid uptake among plant species or cultivars and in the field experiments. It has been found that susceptible varieties to B excess acquired seven times as much B as tolerant ones even if they were grown under the same conditions (Hu and Brown, 1997). Furthermore, Nable *et al.* (1997) found that two barley cultivars grown under identical conditions dramatically differed in boron content (112 *vs* 710 mg kg^{-1}) in the youngest expanded leaf blade. However, the different water use efficiency proposed by Passioura (1997) to justify the same behavior observed in wheat cultivars could not explain these different responses.

Dannel *et al.* (2000) firstly demonstrated the existence of active boric acid transport in sunflower plants grown under low B supply (1 μM B), but not under high boron level (100μM B). They suggested a saturable carrier mediated under low B level and a non-saturable linear diffusion under high B level involving either the root uptake process or xylem loading (Dannel *et al.*, 2000). Similar results were also obtained using the charophyte alga *Chara coralline* (Stangoulis *et al.*, 2001).

In the recent years, experimental evidences have clarified that there are three different molecular mechanisms for boric acid transport from soil solution into root cells and xylem loading, depending on B availability:

1. Passive diffusion across plasma membrane operates under adequate or relatively high B supply;
2. Facilitated transport by non-selective membrane channels (NIP) belonging to the major intrinsic protein (MIP) family operates under limited B supply;
3. Energy-dependent high-affinity transport against concentration gradients, mediated by selective B transporters (BOR) operates in response to low B supply.

Therefore, under adequate or excess B availability, plants showed a passive diffusion across lipid bilayers to B absorption into root cell to satisfy the B requirement of plants. Nevertheless, under limited B availability a facilitated membrane transport of boric acid through MIPs channel and an energy-dependent high-affinity transport system mediated by BOR transporters is required for B transport into the roots and towards xylem (Figure. 2) (Takano *et al.*, 2006; Choi *et al.*, 2007; Tanaka and Fujiwara, 2008; Miwa and Fujiwara, 2010).

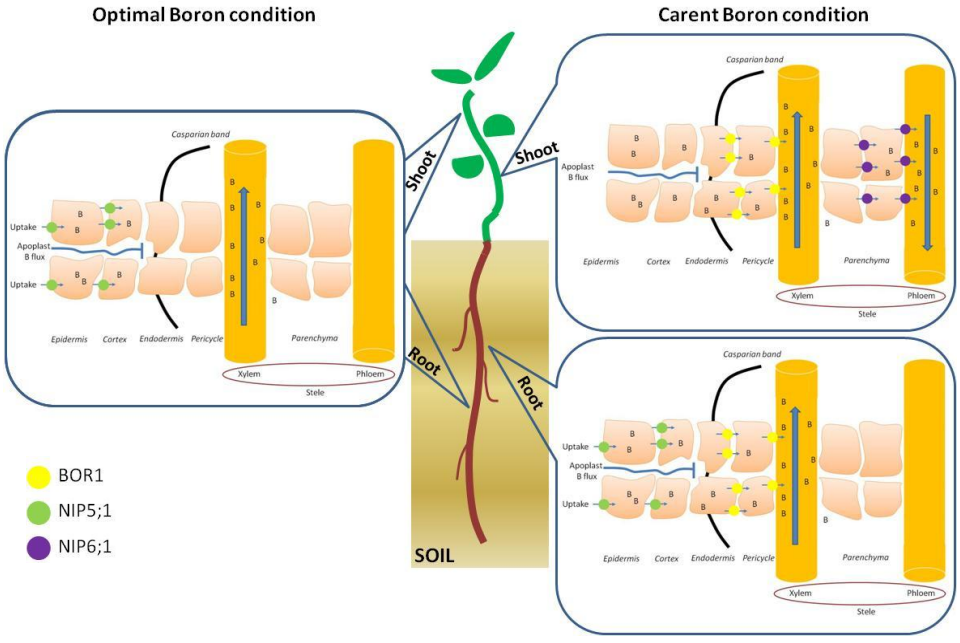


Figure 2. *Model illustrating the different function of boron transporters in whole plant under optimal and deficient B conditions*

1.4.4.1 Facilitated B transport system: the role of aquaporin-like channels

Plant aquaporins are hydrophobic transmembrane proteins with six membrane domains (Tyerman *et al*., 2002) also known as major intrinsic proteins (MIPs). Based on both sequence homology and localization, they are clustered into four subfamilies: plasma membrane (PIPs), tonoplast (TIPs), nodulin 26 like (NIPs) and small basic (SIPs) intrinsic proteins (Chaumont *et al*., 2005; Johanson and Gustavsson, 2002; Johanson *et al*., 2001). Aquaporins are essentially channel proteins which mediate the movement of water and different low molecular solutes such as urea and glycerol (Tsukaguchi *et al*., 1998; Borgnia and Agre, 2001, Kaldenhoff and Fischer 2006), but they are also involved in various physiological processes (Ma *et al*., 2006).

Dordas *et al*. (2000) firstly suggested that aquaporin-like channels were involved in boric acid transport demonstrating that its permeation across the plasma membrane was partially reduced by $HgCl_2$ and phloretin, two channel blockers. These results were later confirmed through *in vivo* experiments on squash roots (Dordas and Brown, 2001). In addition, they demonstrated that some small solutes like urea and glycerol could competitively suppress boric acid uptake up to 54%, confirming the involvement of membrane channels to facilitate boron acid movement. Similar results were also obtained by Fitzpatrick and Reid (2009) using metabolic inhibitors in barley roots. However, the possibility to express aquaporin channel proteins of different species in *Xenopus laveis* oocytes led to improve the knowledge of boric acid absorption. Dordas *et al*. (2000), by expressing a *Zea mays* aquaporin (Zm-PIP1) in *Xenopus laveis* oocytes, firstly observed that their permeability to B increased by 30%. In particular, Zm-PIP1 belongs to PIPs subfamily which includes subgroups PIP1 and PIP2, characterized by very low/null and high water channel activity, respectively (Chaumont *et al*., 2005). The role of PIP1b, PIP2a and PIP2b was also demonstrated by Nuttall (2000) in *Xenopus* oocytes where its expression increased the permeability of oocytes to boric acid. Later, Fitzpatrick and Reid (2009), using yeast complementation assays, demonstrated that HvPIP1;3 and HvPIP1;4 expressions increased boric acid transport in barley roots. These results supported that at least some boric acid flux occurred through a channel-like protein. Recently, two rice genes, *OsPIP2;4* and *OsPIP2;7* have been found to mediate B permeability in yeast and *Arabidopsis*, showing also a role in conferring tolerance to B toxicity (Kumar *et al*., 2014).

Moreover, members of NIPs family have also been involved in B transport. In particular NIPs were firstly localized in the peribacteroid membrane of soybean nodule cells but their subcellular location in non-leguminous plants is not known (Tyerman *et al*., 2002, Chaumont *et al*., 2005). Classified on the basis of the similarity and dissimilarity of their aromatic/argininine (ar/R) region with that of the archetypal

Nodulin26, NIPs is divided into two groups: Group I which possesses the conserved ar/R region of Nodulin 26 and Group II with divergent ar/R tetrad. This latter includes NIP5;1, NIP6;1 and NIP7;1.

To investigate how plants survive under B limited supply, a transcriptome analysis led to identify *AtNIP5;1* gene which codes *AtNIP5;1* protein channel (Takano *et al.*, 2006). It was localized in the plasma membrane of root epidermal, cortical and endodermal cells, especially in the root elongation zone where it was strongly up-regulated under B deficiency. Furthermore, NIP5;1 facilitated boric acid flux in *Xenopus* oocytes by heterologous expression of *AtNIP5;1*. The authors also showed a reduced B uptake together with a severe growth retardation both in shoots and roots in T-DNA insertion *AtNIP5;1* mutant lines under limited B supply (Takano *et al.*, 2006). Later, *OsNPI3;1*, homolog to *AtNPI5;1* has also been identified as boric acid channel in rice (Hanaoka and Fujiwara, 2007). Furthermore, the disruption of *AtNIP5;1* gene caused a higher sensitivity under B limitation (Takano *et al.*, 2006).

The presence of another member of the Group II NIPs involved in B transport, named *AtNIP6;1* under B deficiency has been reported in *Arabidopsis* (Tanaka *et al.*, 2008). *AtNIP6;1* is involved in xylem-phloem transfer of boric acid at the nodal regions, showing the rapid permeation of boric acid but not water. Furthermore, *AtNIP6;1* transcript accumulation is highest in both young rosette leaves and shoot apices but not in roots (Tanaka *et al.*, 2008). They concluded that AtNIP6;1 might play a different role from *AtNIP5;1* in B transport for its tissue specificity.

Schnurbush *et al.* (2010a) demonstrated that *HvNIP2;1* aquaporin could facilitate the B transport when expressed in *Xenopus* oocytes and also it was able to increase the plasma membrane permeability to B in yeast. The control of its expression could limit B toxicity in barley.

Finally, *AtTIP5;1* aquaporin is the only protein belonging to TIPs subfamily involved in B transport pathway possibly *via* vacuolar compartmentation in *Arabidopsis* (Pang *et al.*, 2010) which plays an important role in boron toxicity tolerance.

1.4.4.2 Boron transporters

The isolation and characterization of the *Arabidopsis thaliana* mutant *bor1-1* (high boron requiring), sensitive to boron deficiency was the first evidence that BOR1 could be directly or indirectly involved in B metabolism in higher plants (Noguchi *et al.*, 1997). In particular, the B-uptake analysis indicated that *bor1-1* mutant was unable to tolerate a reduced B delivery to shoots because of impaired xylem loading, showing a severe retardation in plant growth (Noguchi *et al.*, 1997). Thereafter, the

first B transporter, *AtBOR1*, was identified through map-based cloning in *A. thaliana* and characterized as a membrane protein with homology to bicarbonate transporters in animals (Takano *et al.*, 2002). Expressed in the pericycle cells of root stele of *A. thaliana*, *AtBOR1* was responsible for xylem loading and essential for protecting shoots from boron deficiency. Indeed, under low B supply, mutant *bor1-1* showed a lower B concentration in xylem sap than wild type confirming that BOR1 acts as borate exporter to the xylem against B-concentrations. Previously, the same authors had proved that BOR1 was also involved at least in part in the preferential distribution of B to young leaves under a low B supply (Takano *et al.*, 2001).

BOR1 is a member of the solute carrier (SLC4) family of transporters which are classified into three main classes: anion exchangers (AEs), sodium coupled bicarbonate transporters (NCBTs) and borate/boron transporters (BOR-type) (Frommer and Wirén, 2002; Reid, 2014). Databases reported the existence of seven predicted proteins in *A. thaliana* similar to BOR1 which also exhibited strong similarity to expressed sequence tag (EST) clones from diverse plant species, including angiosperms and gymnosperms. This indicated that AtBOR1 belongs to a group of highly conserved membrane proteins in plants (Frommer and Wirén, 2002; Miwa *et al.*, 2013). For many years, *AtBOR1* was considered a borate/chloride anion exchanger and compared to BAND3 (AEs), a prototype anion exchanger of bicarbonate and chloride in red blood cells (Takano *et al.*, 2002). However, the phylogenetic analysis of SLC4 family evidenced that BOR1 shared the same clade with a human bicarbonate transporter related protein, *HsBTR1*, belonging to NCBTs (Frommer and Wirén, 2002; Park *et al.*, 2004). Although sequence similarity between BOR-type and HsBTR1 was very low (23 % amino acid over 60 %), it was higher than that found between BOR1 and AEs (Parker and Boron 2013). Another BOR1 homolog, YNL275 which operates as efflux boron transporter in *Saccharomyces cerevisiae*, has been found, able to maintain the soluble B concentration in the wild type yeast cells 13 times less than their mutant counterparts (Takano, 2002). A low similarity between *AtBOR1* and *ScBOR1p*, a yeast boron transporter, about 32 % over 60 % of the protein sequence was also reported (Reid, 2014).

Although the nature of co-transporter responsible for borate anion outward movement from cytoplasm of plant cells has not been reasonably identified, there is much evidences supporting the hypothesis of ion H^+ as possible driver for borate co-transport in plants (Reid, 2000; 2014). A recent work reported the capacity of BOR-type transporters to produce a concentration gradient in plant cells for which it was necessary the energy source to produce the electrochemical potential. Studies on barley and yeast cells showed that B efflux was unaffected by addition of Na^+, Cl^- or

bicarbonate while increased with increasing H^+ concentration (Jennings *et al.*, 2007; Reid, 2014).

In order to efficiently regulate B transport and maintain its homeostasis, a sophisticated regulation mechanism of BOR1 proteins in response to B availability into the environment was employed by plants. Indeed, under low B supply, BOR1 proteins sorted by the early endosome for recycling into the plasma membrane and there were accumulated; under high B supply, BOR1 proteins were sent to the late endosome and transported to the vacuole for eventual degradation (Takano *et al.*, 2005, 2010). Conversely, the same authors observed that *BOR1* mRNA accumulation was not affected by B availability, suggesting that *AtBOR1* gene was constitutively expressed but its expression was regulated at post-transcriptional level (Takano *et al.*, 2005).

The role of BOR1 as boron exporter for efficient xylem loading under B-limited conditions was also supported by the identification of its localization in the plasma membrane of endodermis cells facing the root stele, or in all the cells of the root on which endodermis was absent (Takano *et al.*, 2010; Miwa *et al.*, 2013).

In the *Arabidopsis* genome there are six BOR1 paralogs. The most similar paralog of BOR1 is BOR2, which encodes an efflux B transporter localized in plasma membrane cells facing the stele. BOR2 is strongly expressed in epidermal cells but not in endodermis of roots elongation zones, complementing the distribution of *AtBOR1*. It is indispensable for root growth and RG-II-B cross linking in cell walls under B-limited conditions (Miwa *et al.*, 2013; Reid, 2014). Another paralog is BOR4 localized into the plasma membrane of the outer side of root epidermal cells, whose overexpression determines an efficient B efflux from roots under toxic B level (Miwa, 2007).

In dicotyledons and monocotyledonsous many BOR1-like genes have been identified. In rice *OsBOR1* expression of efflux boron transporter was identified ,involved in boron uptake and xylem loading (Nakagawa *et al.*, 2007).

Recently, Reid (2014) clearly underlined three main functions of boron transporters: i) Pumping of B into cell walls; ii) Radial transport of B across roots and shoots; iii) Avoidance of toxicity which contributes to maintain B homeostasis in plants.

1.4.4.3 Boron movement in plants

Once B has been absorbed by roots, it was loaded into the xylem and apoplastically transported to shoots via the transpiration stream (Shelp *et al.* 1995). Boron was then accumulated in older leaves without being re-traslocated in many

plant species (Brown *et al*., 2002). For this reason, B deficiency symptoms were firstly evident into the growing root and shoot tissues (Stangoulis *et al*., 2001).

Under adequate B supply, xylem loading occurred by passive mechanisms involving B simple or facilitated diffusion through lipid bilayer and channels, respectively (Dannel *et al*., 2002). On the other hand, under B deficiency, an active transport system via B transporters has been postulated. BOR1 was identified as the first transporter involved in the xylem loading in *Arabidopsis thaliana* (Takano *et al*., 2002), and similar *BOR1* gene was reported in rice (Nakagawa *et al*., 2007), in *Eucalyptus* (Domingues *et al*., 2005). Like, *At BOR1*, *AtBOR2* and *NIP5;1* seemed localized to one side facing the vascular system as a low resistance symplastic pathway involved in B xylem loading (Miwa *et al*., 2013; Reid, 2014). However, NIP6;1 transporter was also involved in B distribution in shoots. In particular, a marked *NIP6;1* promoter activity in phloem region was observed suggesting a specific role of NIP6;1 transporter in B distribution into young growing tissues (Tanaka *et al*., 2008).

However, phloem also plays a role in providing B to sites that do not lose water readily such as both vegetative and reproductive tissues depending on species (Brown and Shelp, 1997; Matoh and Ochiai, 2005). These species commonly showed boron concentrations higher in young leaves compared the old ones under boron deficiency. It has been suggested that the mechanism of B transport through phloem occurred the formation of B-diol complex with sugar alcohols (sorbitol, mannitol or dulcitol),generally used by these species for the phloem translocation of photosynthates (Hu *et al*., 1997). Interestingly, Brown *et al.* (1999) observed that transgenic tobacco plants with elevated sorbitol production had higher ability to transport B by phloem towards the young tissues, compared to plants without sorbitol (Bellaloui *et al*., 2003). Several studies observed that B re-traslocation has important effect on the expression of B deficiency and toxicity symptoms. Nable *et al.* (1997) reported that most species in which B is phloem mobile are susceptible to B toxicity.

Recently, plants such as canola and wheat which did not show nonsugar alcohols but translocates sucrose as its primary photoassimilate, can transport boric acid preferentially to young tissues within the phloem (Stangoulis *et al*., 2010).

1.5 BORON TOXICITY IN PLANTS: CAUSES AND CONSEQUENCES

Boron toxicity is an important disorder in plant causing discoloration of leaves, its typical symptom, but, above all, which seriously affects both yield and quality as a consequence of reduced plant vigor, delayed plant development, decreased number, size and weight of fruits (Paull *et al*., 1992a; Muntean, 2009; Punchana *et al*., 2004). Although B toxicity in plants had been recognized since 1930 by Christensen (1934), its adverse impact was not experimentally confirmed until the early 1980's, when the 17% reduction in barley yield was attributed to high soil boron concentration in South Australia, (Cartwright *et al*., 1984).

The toxic mechanism is poorly understood yet (Nuttall 2000; Reid *et al*. 2004). However, taking into account the B ability to bind compounds with multiple hydroxyl groups in the *cis*-configuration, ribose appeared to be the probable candidate for toxicity-related effects. In particular, *cis* hydroxyls on the ribose side of energy-carrying molecules such as adenosine triphosphate (ATP), nicotinamide adenine dinucleotide (reduced form, NADH), or nicotinamide adenine dinucleotide phosphate (reduced form, NADPH) could be sites of B binding. However, due to the insensitivity of both photosynthesis and respiration to very high B concentrations, it is unlikely that binding to energy-carrying molecules could be considered the actual cause of toxicity (Reid *et al*., 2004). Nozawa *et al*. (2006) identified several ribosomal proteins and transcription factors from *Arabidopsis* which may prevent boron from binding, conferring boron tolerance in yeast. They suggested that B could interfere with transcription and/or translation by binding to *cis* hydroxyls on ribose molecules that are exposed during gene splicing and/or in the t-RNA, thereby protecting transcription and translation (Nozawa *et al*., 2006; Reid, 2010).

1.5.1 SYMPTOMS OF B TOXICITY

Boron toxicity was often confused with spot-type net blotch, a common leaf disease (Brennan and Adcock 2004) and especially at early stages, B symptoms are barely distinguishable from those of other toxic ions in plants. Boron toxicity symptoms vary across crops species. However, its typical visible symptoms are generally correlated with the venation of the older leaves on which burns, chlorosis and/or necrosis appear at the margins and the tips (Oertli and Kohl, 1961). In boron-immobile species these symptoms then spread among the lateral veins towards the midrib (Mengel and Kirkby, 2001) as a result of B accumulation transported through the transpiration stream (Nable *et al*., 1997; Reid *et al*., 2004; Reid and Fitzpatrick, 2009). In dicots which generally have reticulate venation,

toxicity is observed around the leaf margins, whereas in grasses, such as wheat and barley, characterized by parallel-veined leaves, the toxic effect develops black patches in leaf tips where the veins terminate (Roessner *et al.*, 2006). For this reason, leaf burn and necrosis have been extensively used for B toxicity evaluation in different crops (Sutton *et al.*, 2007; Brennan and Adcock, 2004; Torun *et al.*, 2003).

Others specific visible symptom of B toxicity is the reduction of leaf area (Roessner *et al.*, 2006), coupled with leaf cupping, observed in some plant species, probably correlated with the inhibition of cell wall expansion, through disturbance of cross-linking (Suarez, 2012).

Moreover, Cervilla *et al.* (2012) examined different abiotic-stress indicators to select the parameters most indicative of B toxicity in two tomato genotypes, characterized by different sensitivity to B excess. They indicated the O_2•-and anthocyanins level in leaves together with GPX activity, chlorophyll b and proline content as the best indicators for B stress level in tomato plants.

In boron-mobile species (e.g. Prunus, Malus, Pyrus), B accumulation has been observed in developing sinks rather than at the end of the transpiration stream. In these plants the symptoms of B toxicity are expressed as fruit disorders (gummy nuts, internal necrosis), bark necrosis which appears to be due to death of the cambial tissues, and stem die back (Brown and Hu, 1996). In particular, in stone-fruit trees, B toxicity caused the reduction of flower bud formation, poor fruit set and malformed fruit specially poor flavor (Suarez, 2012). In contrast, in rice, a boron-mobile species, B toxicity caused similar foliar symptoms as barley (Bellaloui, 2003).

A direct relationship between B content in leaves and the severity of toxicity symptoms has been demonstrated. Oertli and Roth (1969) reported that the chlorotic/necrotic patches showed much higher B concentrations compared to the surrounding leaf tissues. Furthermore, leaf B concentrations of sensitive and tolerant species have been reported to vary extremely up to ten-folds (Furlani *et al.*, 2003). For this reason diagnosis of B toxicity has been extensively done by tissue B content in leaves and not in shoots and foliar analysis (Reid, 2013).

Moreover, critical toxicity values of tissue B concentrations have been established in many plant species since, B concentrations also greatly varied in relation to different parts or plant tissues and plant developmental stages.

1.5.2 EFFECTS OF BORON EXCESS IN PLANTS

To explain B toxicity mechanisms, many data on the negative impacts of B excess on important biochemical and physiological processes during plant life cycle has been reported.

1.5.2.1 Boron and root system

A primary phenotypic effect of B toxicity is a root growth inhibition often associated with a decreased plant dry weight (Turan *et al.*, 2009) and a B increased level in root tissues. The reduction of root growth has been observed in different crops such as soybean (Kovack and Kleidus, 2008), tomato (Cervilla *et al.*, 2009), wheat (Turan *et al.*, 2009) and grapevine (Ghanati at al., 2008). In particular, B toxicity caused an abnormal cell division in root meristem of broadbean (Liu *et al.*, 2000), and a formation of hypodermis together with a progressive suberin deposition in cortical cell wall of soybean roots (Ghanati *et al.*, 2008). However, the lignification was not considered to be an essential factor for B-induced root growth inhibition in tomato plants (Cervilla *et al.*, 2009). Further, Reid *et al.* (2004) reported a localized inhibitory response to high B concentration in the wheat root tips but not in mature root zones. Boron excess induced cytotoxic effects on root tip cells during mitosis similar to that of colchicine, forming bridges, fragments and stickiness in chromosomes and micronuclei development (Liu *et al.* 2000; Konuk *et al.* 2007). Recently, Aquea *et al.* (2012) reported the molecular basis of root growth inhibition caused by B-toxicity in *Arabidopsis*. They observed that B-toxicity induced the expression of genes involved in abscisic acid (ABA) signaling, ABA response and cell wall modifications, and repressed the expression of genes encoding water transporters, concluding that B-toxicity triggered a water-stress response associated with root growth inhibition. Considering the role of the root system in B excess response, genotypic variation in root elongation has been well used as an indicator of B tolerance (Hayes and Reid, 2004; Choi *et al.*, 2006). Indeed, Choi *et al.* (2007) showed that B tolerance in barley is associated with root morphological changes, leading to an increase in branching and finer root development which allowed a better soil exploitation as result of osmotic adjustment. Recently, Princi *et al.* (2013) reported that short-term treatment to B excess had an evident effect on different root morphological traits. In particular, under B excess, tolerant tomato hybrid showed a longer and thinner root system compared to susceptible one.

1.5.2.2 Boron and photosynthesis process

Although the mechanisms of B toxicity on photosynthesis is still unclear, high boron stress is very damaging for this essential process. Under high boron stress, the edge of the leaf died (Fang, 2001), the photosynthetic area and the chlorophyll content were reduced and consequently the photosynthetic rate (Ardic *et al.*, 2009; Chen *et al.*, 2013; Han *et al.*, 2009; Guidi *et al.*, 2011). A contrasting result was reported only in barley leaves where photosynthesis was not particularly

sensitive to B excess, since it was unaffected by 50 mM and inhibited by only 23% at 100 mM B (Reid *et al.*, 2004). Unver *et al.* (2008) showed a possible role of photosystem II (PSII) Protein D2 to regulate B toxicity in *Gypsophila perfoliata*. Furthermore, Landi *et al.* (2013) found that B excess caused a Chl a/b ratio decreasing together with a down-regulation of PSII photochemical efficiency in cucurbits (*Cucumis sativus* L. and *Cucurbita pepo* L.). In many species, B excess significantly reduced Fv/Fm ratio (maximum quantum yield of chlorophyll fluorescence) which indicated that leaves were photoinhibited (Guidi *et al.*, 2011), condition that can lead to the ROS generation (Velez Ramirez *et al.*, 2011). This event could also explain the decrease in chlorophyll content (Chen *et al.*, 2012; Han *et al.*, 2009) and the chloroplast damage (Papadakis *et al.*, 2004). Furthermore, the inhibition in electron transport rate was also associated to the reduced activity of some enzymes involved in CO_2 assimilation (carboxylase/oxygenase, ribulose-1,5-bisphosphate and fructose-1,6-bisphosphate phosphatase) determining a reduction in NADPH and ATP utilization (Han *et al.*,2009). Recently, Chen *et al.* (2013) investigated protein profiles in leaves of *Arabidopsis* in response to B excess through a proteomic approach. Interestingly, proteins involved in both light and CO_2 fixation reactions of photosynthesis process was affected by B excess, before the appearance of visible symptoms in leaves and the decrease in chlorophyll content, total cell protein, or growth.

1.5.2.3 Boron and antioxidant pathways

Abiotic stress generally promote oxidative stress which caused ROS accumulation, such as hydroxyl radicals (OH•), superoxide radicals (O_2•-) and hydrogen peroxide (H_2O_2), responsible to proteins, nucleic acids and lipids damages, that eventually lead to the cell death (Gill and Tuteja, 2010). Under B toxicity, ROS accumulation in barley (Karabal *et al.*, 2003) and wheat (Gunes *et al.* 2007) has been observed. Boron excess also induced oxidative damage by lipid peroxidation and hydrogen peroxide accumulation in grapevine (*Vitis vinifera*) and *Artemisia annua* (Gunes *et al.*, 2006; Aftab *et al.*, 2010). Since antioxidant molecules such as ascorbate and glutathione (non-enzymatic antioxidant activity) and enzymes such as ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD) were considered an important defense mechanism against free radicals (Sharma *et al.*, 2012), they have been studied in different crops under B-stress conditions (Aftab *et al.*, 2011; Cervilla *et al.*, 2007; Karabal *et al.*, 2003). Boron excess inhibited the formation of glutathione in sunflower (*Helianthus annuus*) leaves (Ruiz *et al.*, 2003) and tocopherol in orange (*Citrus x sinensis L. Osbeck*) where it also boosted ascorbate, glucose and fructose concentrations (Keles *et al.*, 2004). In apple rootstock, glutathione and ascorbate

content increased with increasing B concentrations in the culture medium (Molassiotis *et al.*, 2006). The authors also sustained that a decline in the proline content, able to detoxify ROS, in leaves could contribute to greater lipid peroxidation under B excess (Molassiotis *et al.*, 2006). The non-enzymatic antioxidant activity could have a protective function against oxidative stress induced by B also, in tomato (*Solanum lycopersicum* L.) whereas increased ascorbate pool size, enzyme involved in ascorbate biosynthesis and the enzymes of the Halliwell-Asada cycle were observed (Luis *et al.*, 2007).

Although B-tolerant chickpea (*Cicer arietinum* L.) and basil (*Ocimum basilicum* L.) genotypes seemed to cope with B stress enhancing antioxidant machinery, the signaling and coordination of responses remain unclear yet (Ardic *et al.*, 2009; Landi *et al.*, 2013).

1.5.2.4 Boron and carbohydrate metabolism

Alterations in sucrose levels are highly common in plant responses to various environmental stresses (Rosa *et al.*, 2009), including boron. Several studies showed that B had a variable effect on plant glycosides biosynthesis including sucrose (Dugger and Humphreys, 1960). For example, a decline in glucose in both leaf and root sap of sugar beet under B toxicity was observed (Bonilla *et al.*, 1980). Furthermore, B inhibited the formation of starch from sugar. An increase of reducing sugars (RS) have been also found in the root tip under B excess in soil (Marschner, 1995; McDonald *et al.*, 2003). Recently, the invertase activity appeared to increase within the root tip together with a concomitant increase in RS content, glucose and fructose, in tolerant barley varieties under B toxicity. This change in carbohydrate metabolism would support root development maintaining plant growth under B toxicity (Choi *et al.*, 2007). Recently, genome regions (QTLs) associated with RS content have been detected and mapped at high B supply using a segregant population derived from a cross between B susceptible and tolerant barley cultivars (Huynh *et al.*, 2009). The relationship between B tolerance and high level of RS in the root tip under B excess could confirm the role of RS in B tolerance mechanisms.

1.6 BORON TOLERANCE

1.6.1 EARLY CONSIDERATIONS

Soil amendment such as leaching B with water and application of organic compounds to inactivate or immobilize B in soil has been considered the main approach to solve B toxicity issue for many yeras. However, it appears not practically and economically feasible to be applied on large scale in B toxic areas. On the contrary, the most realistic and potentially effective method to increase crop yields in B-rich soils could be the development of B-excess tolerant genotypes (Nable *et al.*, 1997). Genetic variation for B-excess tolerance has been assessed in many crop species since 80' years and until now (Cartwright *et al.*, 1984; Yau, 2002, Hobson *et al.*, 2003; Schnurbusch *et al.*, 2010b; Bogacki *et al.*, 2013). Therefore, more tolerant varieties can be rather easily breed, offering a most hopeful approach to minimizing decreases in crop yield in areas with high B soil concentration. Further, B tolerant plants provide organic matter that helps the soil retain moisture acting as an excellent food source to support soil microbes as an initial vegetative cover (Reiley and Shry 2000). Therefore, tolerant plants could be the best and eco-friendly approach to recover natural soil conditions and accommodate native vegetation again (Kayama, 2010).

1.6.2 TOLERANCE MECHANISMS REVISITED

Physiological mechanisms related to B excess tolerance are not well understood yet. Tolerance mechanisms in vascular plants include B absorption from soil, B mobility within plant, B accumulation at the end of transpiration stream, tissue B contents, concentration gradient within a leaf (Reid *et al.*, 2004). Boron tolerance model assume i) the existence of binding B compounds once it accumulates to toxic concentrations within the cell; ii) the B compartmentation and iii) an active B efflux by transporters (Hayes and Reid, 2004). Moreover, B accumulation at lower concentrations in tolerant cultivars compared to sensitive ones underlined the predominant role of efflux-type borate transporter(s) in roots rather than internal tolerance mechanisms (B binding complexes or B compartmentation in vacuoles) (Reid, 2007). Taken together the basis of tolerance to B excess postulates a more limited tissue B concentrations involving both the B uptake reduction or the active B efflux, at least partly, from the roots (Reid, 2014).

As discussed previously, *AtBOR1* and *AtNIP5;1* are required for an efficient B uptake when the availability of the microelement in the soil is limited (Takano *et al.*, 2002; 2006). However, it was also shown that under B excess its uptake is mainly regulated through the transcriptional regulation of *AtNIP5;1* (Takano *et al.*, 2006) or

by the endocytosis and degradation of *AtBOR1* (Takano *et al.*, 2005). Besides, *AtBOR1* overexpression does not result in a better plant growth under toxic B concentrations (Miwa *et al.*, 2006). Further, it was also shown that the degradation of *AtNIP5;1* mRNA under B excess is controlled by the 5' untranslated region (UTR) of *AtNIP5;1*, suggesting that both *AtBOR1* and *AtNIP5;1* are not involved in B tolerance (Tanaka *et al.*, 2011).

More recently, many other boron transporters as well as aquaporins have been identified in many plants, for some of which the involvement in B tolerance mechanisms has been proposed (Miwa and Fujiwara, 2010).

Miwa *et al.* (2007) found that *AtBOR4*, one of the six *BOR1* paralogs in the *Arabidopsis* genome, showed a B efflux activity in yeast cells. By using the GFP fluorescence, *AtBOR4* protein was detected on the outer (soil-facing) membranes of root epidermal cells. This localization is important for B directional export to the soil, avoiding high B concentration in growing cells and xylem. *AtBOR4* overexpression improved significantly plant growth under B excess conditions, suggesting that it is exempt from the post-translational *AtBOR1* degradation mechanism, being on the contrary a high-B inducible gene in B tolerance (Miwa *et al.*, 2014). Further, transgenic rice plants expressing *AtBOR4* showed a high tolerance to B toxicity (Kajikawa *et al.*, 2011). The growth enhancement was attributed to the effective B export from the roots, so the B level retained in the optimal concentration within the plant. Thus, the difference in *BOR1* and *BOR4* regulation suggests that complex mechanisms for the perception and control of B homeostasis must exist.

Recently, aquaporin isoforms, involved in water and ion transport, appeared to improve tolerance towards many abiotic stresses (Pang *et al.*, 2010). Indeed, the overexpression of *AtTIP5;1*, a tonoplast aquaporin, resulted in increased tolerance to moderately high B levels in the growing medium being involved in borate compartmentation in the vacuole (Pang *et al.*, 2010). Further, two aquaporin rice genes, *OsPIP2;4* and *OsPIP2;7,* have been found to be involved in B permeability and tolerance (Kumar *et al.*, 2014). Both genes, responsible for exporting B from roots, under B excess were down-regulated in shoots and strongly up-regulated in roots, whose higher expression avoided B toxicity. Furthermore, efflux B assay in roots indicated that ^{10}B was excluded from roots of *Arabidopsis* transgenic plants overexpressing *OsPIP2;4* or *OsPIP2;7* after 1 h of exposure (Kumar *et al.*, 2014).

Recently, a gene encoding a NAC-like transcription factor with a single nucleotide polymorphism between the sensitive and tolerant rice cultivars has been identified using recombinant inbred lines (Ochiai *et al.*, 2011). It was demonstrated that the deletion of the single nucleotide appeared to provide tolerance to B toxicity

in rice by disruption of the gene, which was named BET1 (Boron Excess Tolerant 1), in tolerant cultivars. This mechanism could be independent from B efflux since there were not differences in root and/or shoot B concentrations (Ochiai *et al.*, 2011).

To identify novel mechanism involved in B tolerance, two *Arabidopsis* mutants, defecting in genes related to B excess tolerance have been also studied (Sakamoto *et al.*, 2011). Thus, *heb1-1* and *heb2-1* (*hypersensitive to excess B*) mutants, showing growth defects only under excess levels of B, lacked to encode for two subunits of the chromosomal protein complex known as 'condensin II'. Although both *heb* mutants contained less B than wild-type plants, their sensitivity to excess B was much greater. These findings confirmed the existence of tolerance mechanisms different from the B efflux. The 'condensin II' seemed to act in DNA double-strand breaks amelioration and to maintain the replication process, both functions considered to be required for plant B tolerance (Sakamoto *et al.*, 2011).

1.6.3 TOLERANCE MECHANISMS REVISITED

Crop species have varying ranges at which B concentration is considered adequate, and the threshold between B deficient and toxic level is often very narrow depending on different plant tissues (leaf, root, shoot or whole plant) and different growth stages. Therefore, inside each plant species the evaluation of genotypes for its critical B concentration is important for crop yield and tolerant B selection. Three wide categories of tolerance have been established namely sensitive, semi-tolerant, and tolerant (Ayvaz, 2002). The sensitive species can tolerate 0,5 mg L^{-1} of B while the tolerant ones up to 4 mg L^{-1} (Batar *et al.*, 2009). Apple, beans, figs, grapes and peach are considered the most sensitive crops to B excess, barley, maize, peas, potato, tobacco, and tomato among semi-tolerant while alfalfa, carrot, cotton, sugar-beet and turnip appear the most tolerant (Mengel and Kirkby, 2001). Further, the sensitive plants exhibit a strong reactivity to high or low concentrations of B, while the tolerant ones show adaptability to a wide range of B concentrations without evident growth decreases (Ozturk *et al.*, 2013).

To improve a crop species for B excess tolerance it is necessary to select in the genetic variation for this trait novel genotypes able to adapt to area with B at high concentrations. But what are the reliable criteria for B excess tolerance screening ? The physiological basis of B-tolerance proposed by Nable (1990) postulated that B-tolerant varieties showed a reduced B concentrations in their leaf tissues than sensitive ones, probably due to a lower B uptake into both roots and shoots. Boron tolerance commonly implies little or no evidence of B toxic symptoms, low tissue B concentrations, and high growth or yield under soil-B

excess. Indeed, efficient phenotypic assays for B tolerant screening under controlled conditions include leaf symptom expression, relative root length, shoot dry weight and B concentration in root or leaf tissues measurements (Campbell *et al.*, 1994, Jefferies *et al.*, 2000; Schnurbusch *et al.*, 2008). However, the results concerning B concentration and content in genotypes contrasting for B excess tolerance are not always in accordance. Although tolerant cereal genotypes showed low tissue B concentrations under excessive B supply (Nable, 1988; Bellaloui and Brown, 1998; Rehman *et al.*, 2006), tolerant barley and wheat genotypes with high tissue boron concentrations have been also identified (Yau *et al.*, 1997; Torun *et al.*, 2006). These studies confirmed the wide range of intra specific variation in response to B excess in different crop species, some of which are listed in Table 2.

Crops	Name of line, Cultivar *(origin)*	Reference
Barley	Anadolu (*Turkey*)	Avci (1998)
	Baluchistan (*Pakistan*), ICB 104041 (*Afghanistan*), Tadmor (*Syria*), Tokak (*Turkey*), Walfajr (*Iran*)	Yau (1997)
	Sahara 3763 (*Algeria*)	Nable (1988)
Brassica rapa	WWY Sarson (*Australia*), Local (*India*)	Kaur *et al.* (2006)
Brassica napa	Pactol; Star (*Turkey*)	Ozturk *et al.* (2010)
Durum wheat	ICDW 7674 (*Afghanistan*)	Yau (1997)
	Candeal deGrao Escuro 7746, Senatore Cappelli (*Italy*)	Yau *et al.* (1997)
Bread Wheat	IAC287 (*Brasil*)	Furlani (2003)
	India 126 (*India*), Benvenuto Inca (*Argentina*), Turkey 1473 (*Turkey*), Iraq 22 (*Iraq*), Klein Granador (*Argentina*), Lin Calel (*Argentina*)	Chantachume *et al.* (1995)
	Shi#4414/Crow's (*Syria*)	Yau (1997)
	Greek = G6140 (*Greece*)	Nable (1988)
	Halberd (*Australia*), (Wq * KP)*Wmh/6/12 (*Australia*)	Paull *et al.* (1988)
Lentil	ILL 0213A, ILL 2024 (*Afghanistan*)	Hobson *et al.* (2006)
	ILL 1765 (*Afghanistan*), ILL 5883 (*Syria*)	Yau and Erskine (2000)
Alfalfa	Angel, Caliph, Harbinger, Herald, Paraggio (*Australia*)	Howle *et al.* (2012)
	Cyprus (*Cyprus*)	Paull *et al.* (1992b)
Pea	SA 132, SA 310 (*Afghanistan*)	Bagheri *et al.* (1994)
Rice	IR42, IR46, IR48, IR54, IR9884-54 (*Philippines*)	Dobermann and Fairhurst (2000)
Tomato	Kosaco (*Spain*)	Cervilla *et al.* (2012)
	Losna (*Italy*)	Princi *et al.* (2012)

Table 2 *Boron-toxicity tolerant lines or cultivars in different crop species.*

Further, what is the relationship between B deficiency and toxicity for each genotype ? are the B deficiency tolerant genotypes also susceptible to B excess and *viceversa* ? Furlani *et al.* (2003) reported that IAC287 and IAC60 wheat cultivars showed considerable B efficiency being able to produce the highest shoot, spike and grain dry matter under B deficiency conditions among several tested varieties. In their experiments IAC287 showed also a B excess tolerance, since the typical toxic symptoms were not observed up to 32,4 mM B concentration in growing media.

The genetic variation in response to B excess in crops, such as barley and wheat (Torun *et al.*, 2006; Hayes *et al.*, 2013; Pallotta *et al.*, 2014), lentil (Yau and Erskine, 2000; Kaur *et al.*, 2014), rice (Ochiai *et al.*, 2008), and alfalfa (Bogacki *et al.*, 2013) has been more recently utilized for quantitative trait locus (QTL) analyses. This approach allowed i) to map genome regions including genes involved in B excess tolerance, ii) to understand physiological, genetic and molecular mechanisms of tolerance and iii) to breed B excess tolerant genotypes by MAS (Molecular Assisted Breeding).

1.6.4 QUANTITATIVE TRAIT LOCI (QTL) AND ISOLATING GENES INVOLVED IN B TOLERANCE

One of the first example of QTL analysis showed that the B excess tolerance of 'japonica' rice cultivar was greater than that of 'indica' cultivar due to a major QTL that accounted for the phenotypic variation (Ochiai *et al.*, 2008). This difference was evident even though B content in root and shoot of both tolerant and susceptible rice genotypes did not significantly vary, highlighting the potential role of molecular tools for selecting novel B tolerant genotypes (Ochiai *et al.*, 2008).

Anyhow, quantitative trait locus (QTL) detection has been also useful to isolate genes involved in genetic complex traits. The identification of QTL regions and cloning genes conferring B toxicity tolerance is potentially the major challenge for the development of varieties able to grow in high soil B levels. In barley, four QTL associated with B toxicity-tolerance were detected on chromosome 2H, 3H, 4H and 6H. Thus, *HvBot1*, an *AtBOR1*-like gene, was detected in QTL of chromosome 4H and then cloned (Sutton *et al.*, 2007). It was the first B toxicity tolerance gene identified in plants playing a role in limiting the net B uptake into the root and in the disposal of B from leaves *via* hydathode guttation. It was demonstrated that B tolerance mechanism in tolerant cultivar Sahara was related to an increase in copy number of *HvBot1* gene and abundance of mRNA transcript (Sutton *et al.*, 2007).

Another QTL on barley chromosome 3H was identified to control relative root length at toxic B concentrations having a lesser effect than that of 4H QTL but

operating additively to it. Moreover, a gene encoding a NIP-like aquaporin - *HvNIP2;1* – has been identified in barley and mapped to B tolerance QTL on 6H (Schnurbusch *et al.*, 2010a). Finally, Hassan *et al.* (2010) found that chromosome 2H QTL region encoding a S-adenosylmethionine decarboxylase precursor (SAMDC), involved in antioxidative response, and that yeast overexpressing barley SAMDC was able to grow on excess B medium.

In bread wheat, tolerance to B toxicity was controlled by at least three unlinked *Bo1*, *Bo2*, and *Bo3* genes mapped on chromosomes 4 and 7 (Paull *et al.*, 1991; 1992b). They additively controlled yield and tissue B concentrations under excess B condition (Paull *et al.*, 1992a; Jefferies *et al.*, 2000) and one of genes mapped in 7B was considered to play the main role in crop yield under B toxicity (Nable *et al.*, 1997).

Recently, Pallotta *et al.* (2014) described the identification of near-identical, root-specific B transporter genes underlying the two major-effect QTL for B tolerance in wheat, *Bo1* and *Bo4*. They showed that tolerance to high B concentration was associated with multiple genomic changes including dispersed gene duplication, tetraploid introgression, and variation in gene structure and transcript level. A distinct pattern of gene variant distribution correlated to B levels in soils from different geographical regions was also observed. These findings could support wheat breeders molecular tools to select for the accurate variants of tolerance gene required to specific environments. Thus, the characterization of B tolerance in wheat well highlighted the powerful of the new genomic technologies to define key adaptive processes underpinning crop improvement (Pallotta *et al.*, 2014).

1.7 BORON AND NITROGEN METABOLISM: A *FOCUS* ON NITRATE

1.7.1 NITRATE: SIGNAL AND NUTRIENT

Plants can use different chemical N forms available in the biosphere such as gaseous ammonia (NH_3); nitrogen oxides (NO); mineral form of nitrate (NO_3^-) and ammonium (NH_4^+) ions; and to a lesser extent organic N amino acids and peptides (Miller *et al.*, 2007). The specific N source taken up by plants largely depends on environmental factors, particularly soil conditions. In soil characterized by high pH, NO_3^- is the most abundant form of available N (Maathuis, 2009). In such soils nitrifying bacteria are able to oxide NH_4^+ by degradation of amino-N released from decaying plant and animal materials in NO_3^-. Conversely, this nitrification process is not present in waterlogged, acid or low temperature where a large portion of the N form may remain as NH_4^+ (Forde and Clarkson, 1999).

In a typical aerobic agricultural soil, both NO_3^- and NH_4^+ are present, but nitrate is the predominant N form. As reported by Wolt (1994), in 35 agricultural soil samples, NO_3^- concentration is by about 6.0 mM compared to 0.77 mM reported for NH_4^+. However, the high nitrate levels are usually not maintained because run-off and microbial activities determinate a strong depletion. Further, large seasonal and local variations in NO_3^- soil concentrations due to biotic and abiotic factors can be recorded (Crawford and Glass, 1998), these processes are responsible for spatial heterogeneity in NO_3^- over three orders of magnitude (Miller *et al.*, 2007).

Nitrate is not only a key nutrient, but it is also a "signaling molecule" for many physiological processes of plants (Crawford, 1995). Indeed, it can regulate plant gene expression, C/N metabolism, and growth and development (Krouk *et al.*, 2011; Vidal *et al.*, 2008). In plant grown in nitrate-free conditions, NO_3^- supply leads to modulation of nitrate-transport activity, nitrate-assimilating enzymes such as nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS) (Crawford, 1995; Stitt, 1999). NO_3^- also affects carbohydrate metabolism causing a shift from starch to sucrose syntheses (Tischner, 2000) and increases the accumulation of isopentyladenosine, a cytokinin precursor, suggesting a key role on cytokinin synthesis (Sakakibara *et al.*, 1998). NO_3^- supply modifies resource allocation, growth and development by modulating shoot-root allocation (Stitt and Krapp, 1999) and lateral root growth (Zhang *et al.*, 1999) accelerating senescence and promoting flowering (Marschner, 1995).

In the last decades, genomic, transcriptomic and bioinformatic approaches have outlined a complex regulatory network at transcriptional and post-transcriptional levels of the plant responses to nitrate (Krouk *et al.*, 2010). In particular, NO_3^- together with N metabolites regulates the expression of many genes, involved in a wide range of processes in *Arabidopsis* plants (Figure 3).

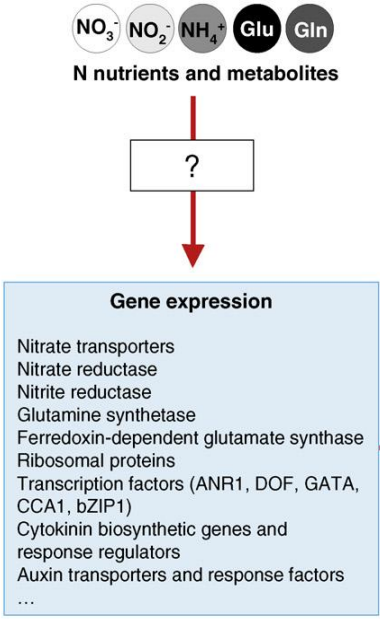


Figure 3. *Gene expression by N supply.*

According to Krouk *et al.*, (2010), up to 10% of the transcriptome is responsive to nitrate (Figure 4) and this anion can be considered a main signal for many genes, since they are still nitrate-regulated in deficient mutants in nitrate reductase, the first enzyme of the nitrate assimilation pathway (Wang *et al.*, 2004).

However, nitrate addition in plants induced different biological functions not only concerning nitrate. Indeed, after 3 minutes of nitrate supply, there was a significant increase in ribosomal proteins and subsequently, of the oxidative pentose-phosphate-pathway suggesting that it induces mechanisms needed to prepare the plants to respond to nitrate (Krouk *et al.*, 2010). In conclusion, the nitrate signaling pathways show complex regulation by transcription factors in the plants and/or by external nitrate availability.

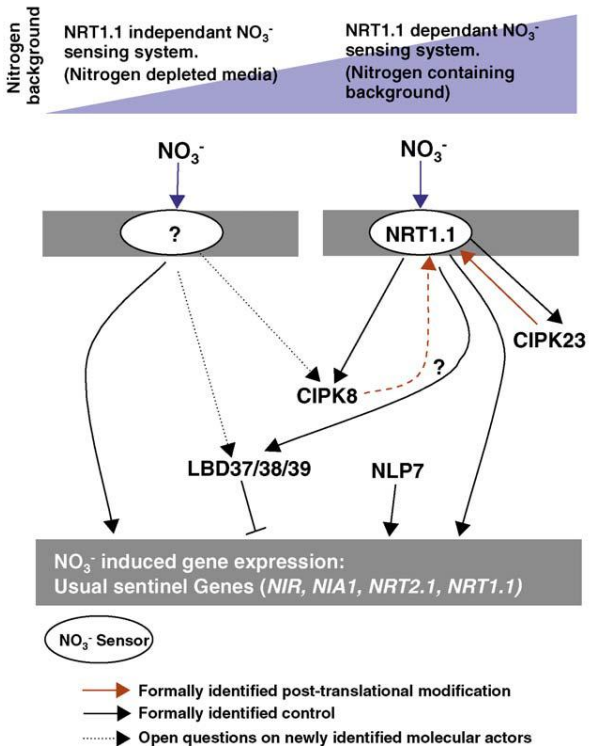


Figure 4. *Model of the signaling molecules acting in nitrate supply by Krouk et al., 2010*

1.7.2 NITRATE UPTAKE, ASSIMILATION AND REMOBILIZATION

The use of nitrate by plants involves several steps including uptake, assimilation, translocation and remobilization. To cope with the spatial and temporal heterogeneity of nitrate concentration in soil solution (Crawford, 1995; Miller *et al.*, 2007), plants have evolved dynamic and flexible root uptake mechanisms. Nitrate uptake has been widely studied in plants (Forde and Clarkson, 1999; Xu *et al.*, 2012). It takes place against the electrochemical potential gradient, driven by electrogenic H^+/NO_3^- symport (McClure *et al.*, 1990), suggesting a strong involvement of pmH^+-ATPase enzyme (Miller and Smith, 1996). Previous data demonstrated that the pmH^+-ATPase activity showed a similar time-course pattern to that of NO_3^- uptake and was also up- and down-regulated by the same signals regulating the NO_3^- influx (Santi *et al.*, 1995; 2003). These results have been recently confirmed in citrus

rootstocks (Sorgonà *et al*., 2010) and along maize roots (Sorgonà *et al*., 2011). It has been proposed that at least three uptake systems for NO_3^- coexist in the plant plasma membrane (Crawford and Glass, 1998). The high-affinity nitrate transport system (HATS, KM of about 50 μM) which operates at low external nitrate concentrations (up to 500 μM) that include two different systems: the constitutive (cHATS) and the inducible (iHATS) (Aslam *et al*., 1992; Glass *et al*., 1995). According to the kinetic parameters, the cHATS displays a higher affinity (Km=6-20 μM) than iHATS (Km=13-79 μM), but a lower nitrate uptake rate (Vmax). The low-affinity transport system (LATS, Km of about 5 mM) which operates predominantly at higher nitrate concentrations (> 1 mM), is both inducible (iLATS) and constitutive (cLATS) (Okamoto *et al*., 2006). It has been suggested that HATS is located close to the root tip, whereas LATS is present in older root parts (Rao-Theertham, 1997). Although both systems, HATS and LATS, operate concurrently, it is difficult to determine the respective roles of each system in root nitrate uptake in crop soil (Miller *et al*., 2007). Malagoli *et al*., (2004) found that HATS had a major contribution to N acquisition in rapeseed. Less information is present on efflux system which is a protein-mediated, passive, saturable and selective process (Aslam *et al*., 1996). Anion channel (s) responsible for NO_3^- efflux must be NO_3^- inducible and often associated with slow growth rates (Nagel and Lambers, 2002; Segonzac *et al*.,2007).

Nitrate transporters (NRTs), belonging to NRT/PTR (peptide transport) family (Tsay *et al*., 2007) and localized in the plasma membrane of root epidermal cells, are involved in root NO_3^- uptake (Kaiser *et al*., 2002). In particular, they comprise two families, the NRT1 (name maintained in the present thesis), recently renamed NPF (Léran *et al*., 2014), that contains more LATS members and the NRT2 containing more HATS members (Figure 5) (Forde, 2000; Tsay *et al*., 2007; Gojon *et al*., 2009).

In *Arabidopsis* NRT1.2 participates in low-affinity uptake among the nine transporter proteins functionally characterized (Huang *et al*., 1999; Krouk *et al*., 2006), whereas, NRT1.1 (CHL1), the most studied nitrate transporter functions as a dual-affinity transporter (Ho *et al*., 2009) and sensor. However, other transporters belonging to NRT1 family are certainly involved in LATS. For example, the NRT1.7 transporter is responsible for phloem loading of nitrate in the source leaf to allow nitrate transport out of older leaves and into younger leaves (Fan *et al*., 2007). The NRT1.9, a plasma membrane transporter expressed in the companion cells of root phloem, may facilitate loading of nitrate into the root phloem and enhance downward nitrate transport in roots (Wang and Tsay, 2011).

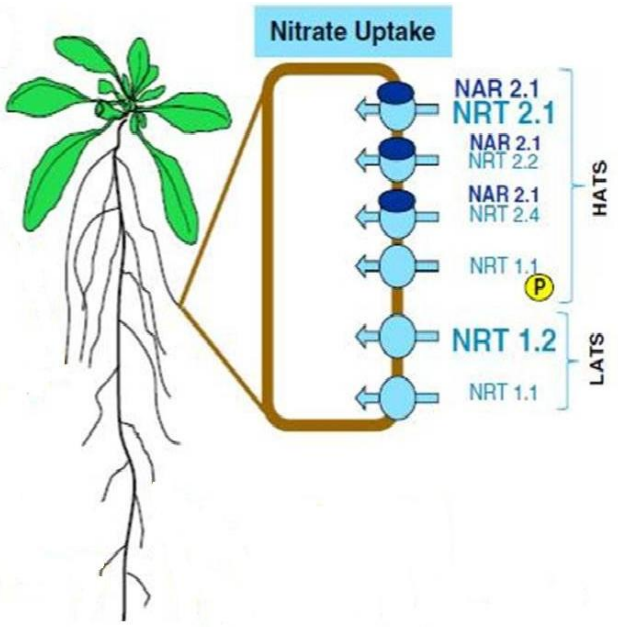


Figure 5. *NRT transporters involved in nitrate uptake by roots*

Homologues of *At*NRT1 have been identified in rice (Lin *et al.*, 2000; Tsay *et al.*, 2007; Plett *et al.*, 2010), maize (Santi *et al.*, 2003; Plett *et al.*, 2010), barley (West *et al.*, 1998; Vidmar *et al.*, 2000; Tong *et al.*, 2005) and tomato (Lauter *et al.*, 1996).

The NRT2 family includes seven genes involved in high-affinity transporters in *Arabidopsis* (Okamoto *et al.*, 2003). In particular, *At*NRT2.1, *At*NRT2.2 and *At*NRT2.4 play an important role in the HATS system (Figure 5). NRT2.1 contributes by 75% of the total HATS activity (Li *et al.*, 2007), whereas NRT2.2, that participates by 20% in normal conditions, has the ability to compensate for a plant defective in NRT2.1, such as the nrt2.1 *Arabidopsis* mutant (Li *et al.*, 2007). Recently, it has been demonstrated that the high-affinity transporter expression is nitrate concentration-dependent. Indeed, below 25 μM nitrate operates NRT2.4 gene, whereas both NRT2.1 and NRT2.2 genes play a major role at concentration higher than 50 μM nitrate (Cerezo *et al.*, 2001). Diverse amino acids have been analyzed for their ability to regulate the expression and the activity of NO_3^- transporters through feedback control such as glutamine (Vidmar et al., 2000). The expression and activity of NRT2.1 requires the concurrent presence of a second protein NAR2.1 (NRT3.1) in *Arabidopsis* (Okamoto *et al.*, 2006; Orsel *et al.*, 2006; Wirth *et al.*, 2007). This complementary role between NRT2 and NAR2 functionality were also found in barley (Vidmar *et al.*, 2000), rice (Feng *et al.*, 2011), maize and sorghum (Plett *et al.*,

2010), soybean (Amarasighe *et al*., 2008), *Medicago truncatula* (Ruffel *et al*., 2008) and tomato (Longo, 2013).

Recently, in maize roots, it has been demonstrated that the regions closer to the root tip early exhibited higher capacity to absorb NO_3^- than the basal regions, because of a higher maximum net nitrate uptake rate (NNUR) and faster induction of the inducible high-affinity transport system (iHATS), the presence of the high-affinity transport system (HATS) also at external NO_3^- concentrations >100 mM and an improved NO_3^- transport because of lower Km values. However, *Zm*NRT2.1 transcript abundances were not spatially correlated with NNUR, suggesting that post-translational effects or NAR2 protein co-expression could be involved (Sorgonà *et al*., 2011).

Once nitrate is taken up into the roots, it can be reduced by assimilatory enzymes. These processes occur in different enzyme-mediated steps and in different intracellular compartments. The first step involves the reduction of nitrate to nitrite by nitrate reductase (NR) enzyme localized in cytosol (Meyer and Stitt, 2001). Then, nitrite is transferred to plastid/chloroplast and here reduced to ammonium by nitrite reductase (NiR). Thus, ammonium is then added to C skeletons to produce different amino acids *via* the glutamine synthetase/glutamine 2-oxoglutarate amino transferase (GS/GOGAT) cycle (Masclaux-Daubresse *et al*., 2010).

The activity of these enzymes can be regulated at transcription, translation and post-translation levels. The site of NO_3^- reduction and assimilation in the plants may vary between the root and the shoot tissues depending on the species, the development stage and the environment (Miller and Cramer, 2004).

In more detail, NR is a cytosolic enzyme which catalyses the transfer of two electrons (reduction) from NAD(P)H to a NO_3^- ion. There are three main forms in plants, NADPH, NADH or both, but roots contain the NADPH and NADH isoforms (Miller and Cramer, 2004). NR is rapidly induced by its own substrate, NO_3^- (Crawford, 1995) and responds rapidly and reversibly to environmental changes (Glaab and Kaiser, 1993). Previous studies reported that there is no correlation between the rate of nitrate assimilation with the increased of nitrate uptake in roots of many species of temperate origin (Andrews, 1986), but it was correlated with the increase of NR activity (NRA) in shoots (Fan *et al*., 2002). However the level of extractable NRA, often under estimated, did not match NR protein or the rate of nitrate reduction *in vivo*, indicating that yet other regulatory mechanisms might exist that modulate the catalytic activity of the protein (Lillo, 1994, and refs. cited therein).

Indeed, posttranslational modulation of NR by protein phosphorylation (inactivating) and dephosphorylation (activating) which represents an important

mechanism for the interactive control of metabolic C and N fluxes has been demonstrated in plant cells (Kaiser and Huber, 1994; Kaiser *et al.*, 2000). The GS-GOGAT is the major pathway of NH_4^+ assimilation in higher plants. GS together with glutamate dehydrogenase (GDH) can combine NH_4^+ with C-molecules. However, the higher Km of GDH (5.8 mM) than GS makes its role for *in vivo* NH_4^+ assimilation unlikely. In roots, two GS isoforms have been found, GS1 and GS2, cytosolic and plastidial, respectively. Thus, GS1 assimilates NH_4^+ derived from the soil or the reduction products of NO_3^- (Ireland and Lea, 1999). Moreover, during the plant life cycle the N forms, stored or included in molecules, can be remobilized, in order to maintain the nutrition of growing organs such as seeds, new shoots and leaves. Release of NH_4^+ in leaf tissues due to nutrient remobilization during senescence, requires that these tissues have the ability to return N to the amino acid pool to be distributed as the plant requires (Liepman and Olsen, 2003). Since carbon skeletons derive from tricarboxyl acid (TCA) cycle, these reactions are essential for C/N metabolism in plants (Lawlor, 2002). In *Arabidopsis*, the time course of nitrate remobilization depends on the different stages of the plant, i.e. vegetative or reproductive (Malagoli *et al.*, 2005; Lemaitre *et al.*, 2008) and the environment (Lemaitre *et al.*, 2008). Finally, genes encoding enzymes involved during N remobilization have been identified and extensively studied (Guo *et al.*, 2004). Moreover, considering the group of enzymes involved in N remobilization the main steps are mediated by glutamine synthetase (GS1), glutamate dehydrogenase (GDH), asparagine synthetase (AS) and aspartate aminotransferase (AspAT) (Masclaux-Daubresse *et al.*, 2010). These last enzymes drive the conversion of glutamine to asparagine and glutamate to aspartate, respectively (Hodges, 2002).

These amino acids, transported *via* phloem, are often distributed to mesophyll cells where they are either stored or utilized for carbon assimilation (Tegeder and Rentsch *et al.*, 2010). The ability of plants to remobilize N into the maturing fruits or grains is of great importance for overall NUE. Finally, all above remobilization enzymes are regulated by many factors such as plant N status, soil N availability, plant hormones (Vidal *et al.*, 2010; Castaings *et al.*, 2011).

1.7.3 BORON AND NITROGEN METABOLISM

Boron affected the N metabolism in many crops. A reduction in NR activity in sunflower seedlings under both B deficiency and toxicity has been reported (Kastori and Petrovic, 1989). Both nitrate reductase (NR) and glutamate dehydrogenase (GDH) activities were affected in leaf and root tissues of barley and wheat under B toxicity (Mahboobi *et al.*, 2002). They found a reduction by about

16% in NR activity in leaf and root tissues of both tolerant and sensitive species, together with an increase (30% in leaf and 81% in root tissues) of GDH activity. This enzyme directly catalyzed the formation of glutamate, the principal precursor of proline biosynthesis, involved in plant defense mechanism (Hong *et al.*, 2000). For this reason, they explained that the increase in GDH activity could represent an adaptive mechanism in both species under B stress conditions.

Response of nitrogen metabolism to B toxicity in tomato has been also investigated (Cervilla *et al.*, 2009). The authors found that glutamine synthase (GS), glutamate synthetase (GOGAT), and GDH increased in tomato leaves under B toxicity, while a significant decrease on NR and NiR activities was observed. They concluded that B toxicity caused an inhibition of nitrate reduction increasing ammonium assimilation in tomato (Cervilla *et al.*, 2009).

Recently, it has been suggested that B excess can also affected nitrate uptake by roots, the first key step of nitrogen metabolism. In sensitive tomato hybrid, boron excess reduced net nitrate uptake affecting the PM H^+-ATPase activity (Princi *et al.*, 2013).

Finally, the possibility of alleviating B stress through improving N fertilization has been evaluated. Aydemir *et al.* (2011) showed that $NH4^+$ supply in lentil and barley had less oxidative damage and yield reduction under B stress in comparison with plants supplied with NO_3^- and urea

1.8 TOMATO CROP

Tomato is one of the major vegetable crop cultivated worldwide, used both for fresh and processed products. Fresh tomato is consumed in salads, and used in many recipes as an ingredient. Processed products include paste, canned tomatoes (diced, crushed and whole), salsa, ketchup and as an ingredient in many condiments.

Tomato was introduced to Europe in the sixteenth century and botanists recognized the close relationship of tomatoes with the genus *Solanum*, identifying it as *S. pomiferum* (Sabine 1820; Luckwill 1943a). Tournefort (1964) has been the first botanist to name the cultivated tomatoes as *Lycopersicum* ("wolf peach" in Greek) by using the multilocular character of fruit as a criterion to differentiate *Lycopersicon* from *Solanum*. Later, Linnaeus (1753) classified tomatoes in the genus *Solanum*, and under the specific name of *Solanum lycopersicum* grouped all the cultivated multilocular forms that Tournefort had described as separate species. One year later Miller (1754) described the genus *Lycopersicum*.

The wild and cultivated tomatoes are native from South America along the west coast and high Andes from central Ecuador, through Peru to northern Chile

and in the Galápagos Islands. The wild cherry tomato is the ancestor of cultivated tomatoes (*S. lycopersicum* var. *cerasiforme*), which is commonly distributed in Bolivia, Colombia, Mexico and other South American countries (Rick and Holle, 1990). Wild tomatoes grow in different western South American habitats, from sea level to over 3,300 m in elevation (Rick 1973; Taylor, 1986). Cultivated tomato were originally domesticated and planted in maize fields by ancient Mexicans (Jenkins, 1948). In the early 1500s tomatoes were propagated to Europe, initially in Italy and Spain, and thereafter became widely spread in the ancient Continent, but until the late 1700s they were not grown and consumed in large quantities.

Actually, tomato is produced on approximately 4 million hectares worldwide, with a total yield of 159.3 million t. The top five leading tomato producing countries are China (36.712 million t), the United States of America (12.953 million t), India (10.261 million t), Turkey (10.134 million t) and Egypt (8.5 million t). In the last ten years, the worldwide tomato production and the land devoted to its cultivation have increased by 46% and 13%, respectively (FAOSTAT 2012). In Italy the total tomato production in 2013 was 4.7 million t; Sicilia, Campania and Calabria are leading regions for fresh tomato production in Italy (Istat, 2013 http://www.istat.it/it/prodotti/banche-dati).

1.8.1 BORON AND NITROGEN METABOLISM

Tomato is an herbaceous annual plant that, with favorable climatic conditions, can behave as biennials and perennials depending on the plant ability to develop secondary growth in basal stems and roots. The plant's lifetime is related to the environmental conditions of every season and its ability to store reserves in the main root and crown. The shoots are initially erect, but later, due to the weight of the branches, the plants become prostrate and in some cases can develop adventitious roots from basal nodes. A strong developed radical system helps the plant anchorage and assures the vegetative and reproductive growth. Wild tomatoes have an indeterminate growth and the main axis of the plant is a sympodium, typical of the genus *Solanum*. Some species are more robust and can develop long branches, to 3–4 m in *S. lycopersicum, S. pimpinellifolium, S. cheesmaniae* and *S. galapagense* and to 6 m in vine forms of *S. habrochaites* (Peralta *et al*., 2008). Tomato plants are formed by a succession of lateral axes with alternate leaves arranged in a 1/3 phyllotaxic spiral in some species or in 1/2 leaf phyllotaxis in others, and the inflorescences are terminal at the end of each sympodial unit (Luckwill 1943b; Danert, 1958).

Cultivated tomatoes (*Solanum lycopersicum* L.) have a semi-determinate or determinate growth habit, with short branches and more compact development.

Leaves are pinnately dissected with 2-6 opposite or sub-opposite, sessile, sub-sessile or petiolate pairs of leaflets. A great interspecific variation in leaf dissection with primary, secondary, tertiary and interjected leaflets can be observed. The degree of leaf dissection is greatly variable among wild tomatoes and sometimes useful to differentiate species (Peralta *et al*., 2008). The first leaf of plantlet is often simple, entire or lobed, or compound with only 1 or 2 leaflets; consecutive leaves are more dissected showing a gradual increase in complexity. The terminal leaflet can be of equal size or larger than primary one. Leaflets are quite variable in size and shape from narrowly elliptic, elliptic to broadly elliptic, ovate or orbicular, the primary leaflets can be sessile to petiolate; the interjected, secondary and tertiary leaflets are usually sessile to sub-sessile.

The plant color is the result of the type, combination, and density of trichomes and varies from bright green in sub-glabrous plants (*S. arcanum* and *S. huaylasense*) to grayish in canescent plants. Glandular trichomes accumulate essential oils that produce the typical smell of tomato leaves that varies considerably among species (Darwin *et al*., 2003). The basic inflorescence is a cyme with different branching patterns (monochasial, dichotomous and polychotomous) and with or without axial bracts. Flowers are typically yellow; the anthers are crosswise joint to form a flask-shaped cone with an elongated sterile tip at the apex (except in *S. pennellii*). The number of flowers per inflorescence axis varies from 4 to 14. The anthers, usually equal in length and straight, are laterally connivent and form a tube. They have a sterile apical appendage and dehisce by introrse longitudinal slits that first appear as small pores and then develop basipetally. The gynoecium is typically bicarpellate (multicarpellate in many cultivars of *S. lycopersicum*), the ovary is superior and globose. Fruits are usually bilocular in the wild species, and bilocular or multilocular in the cultivated varieties. A wide variability of fruit size and shape has developed in different cultivars.

The fruit color derives from the combination of epicarp and sub-epidermic tissue pigments. Four species*, S. cheesmaniae*, *S. galapagense*, *S. lycopersicum* and *S. pimpinellifolium*, members of the "*Lycopersicon*" group have carotenoid pigments (red, orange, and yellow) uniformly distributed throughout the berry. *S. lycopersicum* and *S. pimpinellifolium* have glabrous and typical bright red fruit color by the accumulation of lycopene at maturity, while *S. cheesmaniae* and *S. galapagense* have yellow to orange fruits. Tomato seeds are oval, obovate, or orbicular and flattened laterally with "pseudo-hairs" due to the development of radial wall thickenings of the epidermal cells in mature seeds (Lester 1991; Lester and Durrands 1984) that produce a hairy and silky appearance to the seed surface.

1.8.2 BORON ROLE ON TOMATO GROWTH, YIELD AND NUTRIENT CONTENTS

Among micronutrients, B has a pronounced effect on tomato production and quality in addition to checking various diseases and physiological disorders (Magalhaes *et al.*, 1980). Chude and Oyinloda (2001) reported that tomato plant responses to B soil widely vary among species and genotypes. As reported by Smit and Combrink (2004), 0.16 mg L^{-1} B concentration seemed to be optimal for tomato growth, and levels up to 64 mg L^{-1} did not cause any toxic symptoms. However, an enhanced B supply (B foliar spray at 300 mg L^{-1}) was associated with a less frequent incidence of the physiological disorder shoulder check crack (Huang and Snapp 2004). Davis *et al.* (2003) reported that the dosage of B to tomato grown in river sand, either through the nutrient solution (1 mgL^{-1}) or by foliar spraying (1.87 mg L^{-1}) chelated with mannitol, was associated with increased plant growth and tissue K, Ca and B concentrations. In particular, foliar B spraying significantly enhanced fruit B and K concentrations in comparison with no B supply, indicating that B was firstly translocated from the leaves to the fruit and secondly that B is also involved in K translocation within the plant. An increase in Ca, Mg, Na, Zn and B uptake in the root zone under higher B levels has been reported by Smit and Combrink (2004). Further, B application was associated with an increased N uptake by tomato in field, but not in hydroponic culture (Davis *et al.*, 2003). On the contrary, a suboptimal B supply may considerably reduce fruit set, especially without any support for tomato pollination (e.g. vibration) (Smit and Combrink 2005). Regardless of culture method, Farzaneh *et al.* (2011) analyzed the effect of different nitrogen (100, 200, 300 and 400 mg L^{-1}) and B (0.5, 1.0, 1.5 and 2.0 mg L^{-1}) concentrations and their interaction on yield, shoot and root dry weights and nutrient leaf concentrations in tomato cv. Rio Grande grown hydroponically. The results showed that both simple and interactive effect of nitrogen and B were significant; indeed the highest yield and root dry weights were obtained with 200 and 1.0 mg L^{-1} of N and B, respectively, while the highest shoot dry weight was measured at 300 and 1.0 mg L^{-1} of N and B, respectively. They concluded that 200 mg L^{-1} N and 1.0 mgL^{-1} B in nutrient solution was recommended in tomato to achieve higher yield and fruit quality in hydroponic culture (Farzaneh *et al.*, 2011).

More recently, in field experiment the effect of B concentration on growth and yield of Rio Grande and Rio Figue tomato *cvs.* were reported (Naz *et al.*, 2012). Different B doses (0, 0.5, 1.0, 2.0, 3.0 and 5.0 kg ha^{-1}) were added maintaining constant N, P and K doses (150, 100, 60 kg ha^{-1}). Boron showed a significant effect on tomato growth and yield and 2 kg B ha^{-1} resulted in maximum number of flower clusters per plant, fruit set percentage, total yield, fruit weight loss and total soluble

solid. In particular, maximum number of flower clusters per plant, fruit set percentage and total yield were recorded in Rio Grande. Another important aspect related to B nutrition in tomato is the interaction between B and salinity or water stress. According to Ben-Gal and Shani (2002; 2003), under concurrent stresses, the extent of growth limitation is determined by the factor imposing the most severe stress and not by an additional effect of both restrictive factors. Hence, a dominant-stress-factor model following the Liebig-Sprengel law of the minimum may be used to describe the simultaneous B and salinity or B and water shortage effects on tomato.

1.8.3 BORON TOXIC EFFECTS ON TOMATO PLANTS

In agriculture, both deficient and toxic B levels in soil impair plant growth, resulting in the reduction of crop yield and quality. According to Alpaslan and Gunes (2001), 5 mg kg^{-1} soil B concentrations or higher are expected to impose B toxic symptoms. In general B excess causes in tomato, besides in all species studied so far, reduced vigour, delayed development, leaf burn (chlorotic and necrotic patches in older leaves). However, this reduction is lower compared with that of other sensitive species, such as cucumber, due to a B-exclusion mechanism in the tomato root system (Alpaslan and Gunes, 2001). Despite this B-excluder ability, when B concentrations increase the internal B concentration in plant tissue increase too (Gunes *et al.,* 1999; Alpaslan and Gunes, 2001; Cervilla *et al.,* 2007) depending on tomato cultivars (Cervilla *et al*., 2007). It was also observed that when B excess occurs together with salinity (stress for which tomato is considered a relatively tolerant species), plant tissues B concentration is lower than that of plants exposed to the same B level without salt stress (Alpaslan and Gunes, 2001; Ben-Gal and Shani, 2002). This phenomenon in tomato was explained as a result of the direct link between B uptake and transport through transpiration flow. Effectively, the presence of salinity caused a transpiration decrease and consequently a reduced B uptake (Alpaslan and Gunes, 2001; Ben-Gal and Shani, 2002). In this respect, the cell membrane permeability of tomato leaves did not differ significantly under B excess compared to the control, while in plants concurrently exposed to both stresses (B-excess and salinity) the cell membrane permeability increased (Alpaslan and Gunes, 2001). Differently from vegetative growth, tomato fruit yield and quality seemed to be not particularly affected from relatively high B doses (64 mg L^{-1}) (Smit and Combrik, 2004). Previous observations reported limited decrease in tomato production, indicating also that B excess result in transpiration and water use decreases (Ben-Gal and Shani, 2002).

However, despite the importance of B toxicity for crop productivity, the mechanism by which plants respond to B-excess is still not completely understood so that further investigations are needed. In this sense, one of the most common studies in plants exposed to abiotic stress is to determine the most reliable biochemical, physiological and molecular markers of tolerance for their use in selection among different varieties or in segregant populations. Notably, among the biochemical indicators oxidative stress parameters and osmo-protective compounds appeared to be the most widely used for this purpose (Juan *et al.*, 2005; Sánchez-Rodríguez, 2010). In tomato, significant increase in H_2O_2 concentration and lipid peroxidation accompanied by higher non enzymatic antioxidant activity (ascorbate and glutathione) with B excess (2 mM) in culture medium were found (Cervilla *et al.*, 2007). In particular, antioxidant enzymes activity (CAT, APX and SOD) as well as one of enzymes involved in ASA regeneration (MDHAR, DHAR and GR) significantly increased following a B-excess treatment. In this respect, more recently the rise of O_2^- and H_2O_2 levels as a first indication of high B concentrations in tomato leaves was confirmed, followed by proline and anthocyanins increased levels and higher GPX activity, a best marker of the phenolic metabolism activation (Cervilla *et al.*, 2012). This implies that the ROS levels in the leaf may constitute a reliable parameter to evaluate the degree of B stress in tomato and as well as to develop models that could help to prevent the damage determined by B toxicity in tomato.

AIMS AND OBJECTIVES OF RESEARCH

Boron (B) is an essential micronutrient for plants, and the importance of its application in intensive cropping systems is well recognized. On the other hand, B in excess is toxic occurring in soil naturally or due to over-fertilization and/or irrigation with water rich in B. In some Mediterranean regions, B contamination of groundwater represents a serious constraint to both agriculture and drinking water. However, both of these stress conditions severely reduced crop yield and quality worldwide and their concentration range between B deficiency and toxicity is generally very narrow, differing among crops. A typical symptom of B toxicity is the appearance of chlorotic and/or necrotic spots at the margins and tips of older leaves which can be used to aid diagnosis. Although many evidences point out that several key cellular processes are sensitive to B excess, the molecular mechanisms of B toxicity are not fully understood. Moreover, since B toxicity is more difficult to be managed in cropping systems, it is best dealt with by using B-tolerant varieties.

Remarkable insights into the potential of tolerant plants to avoid B-toxicity are described. The B tolerance is species- specific and it is commonly associated with the ability to maintain a low B concentration in shoots. In *Arabidopsis*, B tolerance was found to be associated with the presence of BOR channels, which are necessary for B extrusion from the cytoplasm. Two groups of genes appear to regulate B uptake and transport in plants: i) BOR1, a B efflux transporter involved in xylem loading in *Arabidopsis* under B deficiency; and ii) nodulin-like intrinsic proteins (NIP) which are candidate channels for the membrane transport of boric acid. Recent papers demonstrated that both these channels, BOR 1 and NIP, are more important in B-deficiency. Indeed, under high B levels, *BOR1* is degraded *via* endocytosis and its overexpression does not improve plant growth. Conversely, BOR4, a B efflux transporter from the roots to the soil, is considered the most important efflux-type transporter able to confer B-tolerance to plants under B-toxicity.

Hence, the root system is not only a recurring target of B excess, but recent findings suggest the importance of root morphology in B-tolerance mechanism in barley.

In this context, the aim of PhD thesis has been focused on the root system responses to B-excess as well as on the role played in tolerance mechanisms in tomato genotypes contrasting in B-sensitivity. Tomato is one of the most important vegetable crop in the Mediterranean basin for both cultivated area and productivity.

Three important topics were studied:

1) Long- and short term responses of root form and function of two tomato genotypes with different sensitivity to B toxicity: 'Ikram' (sensitive) and

'Losna' (moderately tolerant). Boron excess and nitrate interaction with particular emphasis on several biochemical and molecular aspects of anion uptake were also analyzed;

2) Short-term antioxidant responses of two tomato root systems with different sensitivity to B toxicity;
3) Tomato response to boron excess: the role of grafting and root morphology.

CHAPTER 2 BORON EXCESS ON TWO TOMATO HYBRIDS: LONG- AND SHORT TERM EFFECTS ON ROOT FORM AND FUNCTION

2.1 MATERIALS AND METHODS

2.1.1 PLANT MATERIAL AND GROWTH CONDITION

Tomato (*Solanum lycopersicum* L., Ikram and Losna genotypes, kindly provided by *Syngenta,* Italy) seeds, surface sterilized for 10 min in 10% (v/v) sodium hypochlorite solution and rinsed with deionized water, were pre-germinated at 24°C in darkness on filter paper moistened with 0.5 mM $CaSO_4$ for 5 days. Individual seedlings, selected by uniform size, were transferred in cell flats (cell size, 4 cm × 4 cm × 5 cm) filled with silver sand and then placed in a controlled environmental chamber at 25°C with a 16-h photoperiod, a photon flux density of 350 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 70 % RH. The seedlings received a complete nutrient solution containing KNO_3 (6 mM), $NH_4H_2PO_4$ (1 mM), $MgSO_4$ (2 mM), $Ca(NO_3)_2$ (4 mM); KCl (50 μM), H_3BO_3 (25 μM), $MnSO_4$ (2 μM), $ZnSO_4$ (2 μM) $CuSO_4$ (0,5 μM), $(NH_4)_2Mo_4$ (0,5 μM) and Fe-EDTA (20 μM), pH 5.8, for 7 days.

Afterwards, seedlings were transferred into a growing unit containing 4.3 L of aerated nutrient solution having the same above composition, for 7 day. The pH was adjusted to 5.8 with 0.1 N KOH and the nutrient solution was replaced twice a week.

2.1.2 LONG AND SHORT TERM BORON EXPERIMENTS

For long term experiment, tomato seedlings (19 d-old) were transferred to aerated nutrient solution having the same above composition adding by 25 (control) or 320 or 640 or 1280 μM H_3BO_3 for 7 days. Then, root morphological analysis, chlorophyll and boron content were evaluated.

For short term experiment, tomato seedlings (19 d-old) were transferred to aerated N-free nutrient solution (0.5 mM $CaSO_4$) for 24 h, in order to reach the nitrate starvation. After that, tomato seedlings were transferred to aerated nutrient solution and exposed to 200 μM NO_3^- and 25 (control) or 320 or 640 μM H_3BO_3 for 0, 4, 8, 24, and 48 h. Then, root morphological analysis, net NO_3^- uptake and H^+-ATPase assay, membrane potential measurements and gene expression analysis were carried out.

2.1.3 MORPHOLOGICAL ANALYSIS

Five seedlings of each genotype were collected, divided into roots and shoots, for each B treatment of the long-term experiment and for both each exposure period

and B treatment for the short-term one. Total root length (cm), volume (cm^3), and superficial area (cm^2) of the tomato roots were determined by staining with 0.1% (v/v) toluidine blue for 5 min and then image was captured, after 7 days for long boron treatment and at 0, 4, 8, 24 and 48 h of short boron treatment, by scanner and analyzed using WinRhizo software (WinRhizo STD 1600, Instruments Régent Inc., Canada). Shoot (SDW, g) and root (RDW, g) dry weights were determined after oven-drying at 72 °C for 48 h. Finally, root length ratio (RLR, root length/ whole plant dry weight, cm g^{-1}), root mass ratio (RMR, root dry weight/whole plant dry weight, g g^{-1}), root fineness (F, root length/root volume, cm cm^{-3}) and root tissue density (TD, root dry weight/root volume, g cm^{-3}) were calculated according to Sorgonà *et al.* (2011).

Shoot and root growth rates (SGR and RGR, g DW day^{-1}, respectively) were also analyzed by linear regression using the increase of the biomass over time for short term boron treatment.

2.1.4 CHLOROPHYLL CONTENT

Relative absorbance measurements using a SPAD-502 chlorophyll meter (Konica Minolta Sensing, Inc., Japan) were taken in tomato plants (21 d-old) from the lower, middle and upper true leaves of the fully expanded frond. SPAD readings were collected after 7 days of B exposure and the SPAD readings from the lower, middle and upper of the leaves were then averaged for each tomato plant.

2.1.5 BORON CONTENT

Leaf and root samples were collected after 7 days of B exposure, rapidly washed with deionized water and dried at 80°C. The total B concentration was analysed after digestion of 0.15 g dry and milled leaf or root material with a mixture of HNO_3 (98%) and $HClO_4$ (30%) at 230°C for 1 h. Boron was determined according to Azomethine-H method (Wolf, 1974), the absorbance was read by spectrophotometry at 420 nm and the concentration was expressed as g $(kg\ DW)^{-1}$.

2.1.6 NET NO_3^- UPTAKE ASSAY

The net nitrate uptake rate (NNUR) was defined as net influx across the plasma membrane (Lainé *et al.*, 1995).Three tomato starved seedlings (19-d-old) were collected for each B treatment and exposure period and their intact roots were rinsed with 0.5 mM $CaSO_4$ for 20 min. The seedlings were then immersed in 40 mL of continuously aerated nutrient uptake solution containing 200 μM KNO_3 and 0.5 mM $CaSO_4$ at pH 6.0. Sample solutions were taken from the uptake solution at 5 min intervals over a 50 min period and nitrate concentration was measured spectrophotometrically at 210 nm (Goldsmith *et al.* 1973) using a UV-Vis

spectrophotometer (Perkin-Elmer, Norwalk, CT, USA). The NNUR was calculated from the linear phase of the nitrate depletion curve and expressed as μmol NO_3^- g^{-1} FW h^{-1}.

2.1.7 H^+-ATPase assay

2.1.7.1 Isolation of plasma membrane vesicles

Plasma membrane (pm) vesicles were isolated from tomato roots using a small-scale procedure from Giannini *et al.* (1988) modified by Santi *et al.* (1995). For each B treatment and exposure period of short term treatment, tomato roots (1-2 g) were homogenized in extraction buffer (250 mM sucrose, 10 % (v/v) glycerol, 10 mM glycerol-1-phosphate, 2 mM $MgSO_4$, 2 mM EDTA, 2 mM EGTA, 2 mM ATP, 2 mM DTT, 5.7% (w/v) choline chloride, and 25 mM BTP buffered to pH 7.6 with MES, and 1 mM PMSF, and 20 mg/mL chimostatin freshly added before homogenization), filtered and centrifuged twice at 12,700 g for 3 and 25 min, at 4°C. The suspension was layered over a 25/38% discontinuous sucrose gradient (10 mM DL-α-glycerol-1-phosphate, 2 mM $MgSO_4$, 2 mM EGTA, 2 mM ATP, 1 mM PMSF, 2 mM DTT, 20 mg/mL chimostatin, 5.7% (w/v) choline chloride, 5 mM BTP buffered at pH 7.4 with MES) and centrifuged at 12,700 g for 60 min at 4°C. The vesicles, banding at the 25/38 % interface layers, were collected and centrifuged at 14,000 g for 45 min at 4°C. The pellets, resuspended in a medium (20% glycerol (v/v), 2 mM EGTA, 2 mM EDTA, 0.5 mM ATP, 1 mM PMSF, 2 mM DTT, 20 mg/mL chimostatin, 5.7% (w/v) choline chloride, 5 mM BTP buffered at pH 7 with MES), were immediately frozen in liquid N_2 and stored at -80°C until use.

2.1.7.2 pmH^+-ATPase activity

ATP-hydrolyzing activity was determined by measuring the release of inorganic phosphate, as described by Forbusch (1983). The assay medium (0.6 mL) contained 50 mM BTP-MES pH 6.5, 5 mM $MgSO_4$, 5 mM ATP, 0.6 mM Na_2MoO_4, 100 mM KNO_3, 1.5 mM NaN_3, 0.01% (w/v) $Brij_{58}$, with or without 100 μM vanadate (V_2O_5), an inhibitor of P-type H+-ATPase (Sze 1985). Sodium azide (NaN_3, 1 mM) and potassium nitrate (KNO_3, 150 mM) were used as selective inhibitors of mitochondria and tonoplast H^+-ATPase, respectively. The difference between these two activities was attributed to the pmH^+-ATPase. The reaction was started adding 0.5-1.5 μg of membrane protein and stopped after 30 min by a solution containing: 0.6 M HCl, 3% (w/v) SDS, 3% (w/v) ascorbic acid and 0.5%

(w/v) ammonium molybdate at 2°C. The pmH^+-ATPase activity was expressed as nmol P_i μg $protein^{-1}$ h^{-1}

2.1.7.3 Protein assay

Total soluble protein was estimated according to Bradford (1976) using bovine serum albumin as standard.

2.1.8 MEMBRANE POTENTIAL MEASUREMENTS

Measurements of membrane potential were performed on mature cortical primary root cells (at 1 cm from the tip) of intact tomato plants using standard microelectrode techniques. For the electrode impalement, root of tomato seedlings (7 days old), previously starved with 0.5 mM $CaSO_4$ for 24 h, were placed in a plexiglass chamber and perfused with bathing solution containing 5 mM MES, 0.5 mM $CaCl_2$ and 0.05 mM KCl, adjusted to pH 6.0 with NaOH. Membrane electrical potentials were measured with single-barreled microelectrodes. Glass microelectrodes were filled with 200 mM KCl using a 70 mm long Microfil needle (WorldPrecision Instruments Inc., Stevenage, UK) and reference salt bridges filled with 200 mM KCl in 2% agar. The reference electrode was kept in the perfusion chamber, close to the root; while the impalement was performed with a micromanipulator into mature epidermal cells. The voltage differences (mV) between the inside of cell and external bathing solution were than measured. The values from -90 to -140 were considered to define a successful cell microelectrode impalement and measurement. The experimental setup was divided in two steps: in the first one, the membrane potential of nitrate-starved genotypes was measured using 200 μM NO_3^- and/or 320 μM B as perfusion solution either at 0 min (Steady-State) or 30 minutes (depolarization or hyperpolarization); in the second step, membrane potential was recorded in nitrate-starved genotypes grown with 10 μM coumarin or 2 mM orthovanadate (stimulator or inhibitor of plasma membrane H^+ATPase, respectively) for 4 h and then transferred to chamber to measure the steady-state potential, and subsequently perfused with 320 μM H_3BO_3 for 30 minutes.

2.1.9 GENE EXPRESSION ANALYSIS

2.1.9.1 RNA extraction

Tomato (*Solanum lycopersicum* L., Ikram and Losna) seeds, surface sterilized for 10 min in 10% (v/v) sodium hypochlorite solution and rinsed with deionized water, were pre-germinated at 24°C in darkness on filter paper moistened with 0.5 mM $CaSO_4$ for 5 days. Seedlings selected for uniform size were transferred in cell flats filled with silver sand added by a complete nutrient solution and placed in a controlled environmental chamber at 25°C with a 16-h photoperiod, a photon flux density of 350 μmol·m^{-2}·s^{-1} and 70 % RH. Afterwards, seedlings were transferred into a growing unit containing 4.3 L of aerated nutrient solution having the same above composition, for 7 day. The pH was adjusted to 5.8 with 0.1 N KOH, the nutrient solution was replaced twice a week. Before treatments, 19 d-old seedlings were transferred to an aerated N-free nutrient solution for 24 h and then transferred in the same nutrient solution containing 200 μM NO_3^- and 25 (control) or 320 μM or 640 μM H_3BO_3.

Roots from Ikram and Losna seedlings grown in hydroponic culture were sampled at 0, 4 and 8 h from treatments (200 μM NO_3^- *plus* 25, 320, 640 μM H_3BO_3). Total RNA was isolated from 100 mg of fresh root tissue using RNeasy Plant Mini Kit (Qiagen, Milano, Italy), following the protocol provided by the manufacturer, adding also a DNAse treatment by using the Deoxyribonuclease I Amplification Grade (Invitrogen, Life-Technologies). RNA quality and quantification was assayed by NanoDrop 2000 (Thermo Scientific).

2.1.9.2 Reverse Transcript-PCR

RT-PCR was performed in order to detect *NRT2.1, NAR2.1, BOR1, BOR4, NIP5;1, LHA1* and *LHA8* from each treatment and genotype. A tomato ubiquitin was utilized as constitutive standard control. For each treatment, 1 μg of total RNA in 25 μl reaction (QIAGEN OneStep RT-PCR kit), was used optimizing both efficient reverse transcription (cDNA synthesis) and specific amplification. For each of these reactions, a set of different numbers of cycles ranging between 25 and 35 was tested to choose those corresponding to the exponential phase for each gene. Each cycle consisted of a 30 s denaturation at 94°C, a 45 s of annealing at 64°C and a 60 s extension at 72°C; a 30 min reverse transcription at 55°C, a 15 min hot start at 95°C at the beginning of the reactions and finally a 10 min extension at 72°C were performed. Primers for *NTR2.1* (accession number: NM001279334), *NAR2.1* (XM004236225), *BOR1* (NM004241450), *BOR4* (XM004235670), *NIP5;1*

(NM001287359), *LHA1* (NM001247846) and *LHA8* (AF263917) were designated to amplify specific fragments, tomato ubiquitin gene was the internal standards (Table 3). The PCR products were electrophoresed in 1.5% agarose gel, stained with ethidium bromide.

Gene	Forward	Reverse
SlNRT2.1	GGGATCATTGCTGCCACATT	ACCGAATTTCTTTGCTGCGT
SlNAR2.1	GCTGACCACAAAGCAGGAGTATTG	TCAAGGACCACTTGAGCGTGAG
SlBOR1	TGCTACAAGCAAGACTGGACTGG	GCACTGCAGTTATACTTCCATCGG
SlBOR4	GCCTTAAAGAGCAGGAAAGCAAGG	TGAAGCTTCTCATCCAGCCTGTG
SlNIP5;1	AGCTCCTCATACCTGTCTTGCAG	CCACGAATTCAGCTCCCAACTTTC
LHA1	TCGAAGTTGGTCGTTTGTGGA	GAGTTGACCAACGGTGGAACT
LHA8	TTAAGAGGCTGCAGGAGAGG	GGGTCGTGACTTCTTTCGTC
Ubiquitin	GGACGGACGTACTCTAGCTGAT	AGCTTTCGACCTCAAGGGTA

Table 3. *Specific forward and reverse primer sequences (5'-3' oriented) used in semiquantitative PCR expression analysis of the genes under investigation*

2.1.9.3 Quantitative RT-PCR

A first-strand cDNA was synthesized from 1 μg of the total RNA (QuantiTect reverse Transcription Kit), using an optimized RT Primer mix of oligo-dT and random primers as suggested by the Qiagen manufacturer. The real-time PCR (qPCR) was performed on 7500 Real-Time PCR System (Applied Biosystems, Life Technologies) using SYBR Green master mix (Applied Biosystems, Life Technologies) according to the manufacturer's instructions. The qPCR were carried out starting from 2 min at 95 °C (initial denaturation), then for 40 cycles consisting of 30 s at 94 °C, 30 s at 60 °C and 1 minute at 72 °C. Three replicate experiments for each B-concentration and genotypes were carried out. Specific primers for *NTR2.1*, *NAR2.1*, *BOR1*, *BOR4*, *NIP5;1*, *LHA1* and *LHA8* were reported in Table 3, tomato ubiquitin gene was the internal standards. The sequences utilized for designing the primers showed different percentage of identity with the reference genes of other plants; *NRT2.1* (NM001279334) showed 87%, 86% and 84% identity to *NRT2.1* from potato, *Arabidopsis* and tobacco, respectively (Ono *et al.*, 2000), *SlNAR2.1* showed 90% identity to potato *NAR2.1* gene, *SlNIP5;1* showed 87% and 56% identity to *NIP5;1* from tobacco and potato, *LHA1* showed 89% identity to potato and tobacco ATPase-1 genes and *LHA8* showed 96% and 87% identity to

Arabidopsis thaliana ATPase *HA8* gene (AT3G42640.1) and tobacco ATPase (*pma3*) gene.

The qPCR results were analyzed by the $2^{-\Delta\Delta Ct}$ comparative method as previously described in the Real-time PCR Application guide (BioRad) and also by Livak and Schmittgen (2001). Based on the fluorescence logarithmic graph, the fitting threshold was chosen calculating the Ct by 7500 System SDS software, RQ Study Application (Applied Biosystems). This method can detect relative changes in gene expression, where ΔCt is the difference in threshold cycles between target (Ct sample) and reference (Ct ubiquitin) genes. The ΔCt of each sample was than normalized (adopting the ΔΔCt) to the calibrator (time 0 for each boron treatment was considered) to account for variability in original concentration and quality of the total RNA, and the conversion efficiency of the reverse transcription reaction.

2.1.10 STATISTICAL ANALYSIS

In all the experiments a randomized block design was adopted. All data were evaluated for normality and homogeneity of variances by Kolmogorov-Smirnov and Levene median tests, respectively. Root and shoot growth rates were estimated by linear regression, and the slope was used to determine differences between tomato genotypes (ANOVA, Tukey's test, $P<0.05$). Root morphological, SPAD, PM H^+-ATPase activity, boron content and membrane potential data were analyzed by two-way ANOVA comparing genotypes and treatments, and means were separated by Tukey's Honestly Significant Difference (HSD) test ($p < 0.05$) using Systat software (Systat Software Inc, Chicago, USA).

The NNUR for each tomato genotype at different B concentration was described by non-linear regression using the nonlinear equation described Abenavoli *et al.* (2001).

All gene expression data were analyzed by one-way ANOVA comparing among treatments and genotypes, and means were separated by Tukey's Honestly Significant Difference (HSD) test ($p \leq 0.05$).

2.2 RESULTS

2.2.1 LONG- AND SHORT TERM BORON TOXIC TREATMENTS

After 7 d of exposure (long term treatment), B differently inhibited root elongation in two tomato genotypes not previously exposed to B. Indeed, Ikram showed a significant decrease in total root length (TRL) already at 320 μM B compared to Losna and this reduction was also maintained at 640 μM (26 and 29 %, respectively), disappearing at highest concentration where two genotypes showed a similar inhibitory response (Table 4).

Table 4. *Morphological parameters of two tomato hybrids exposed to different boron level for 7 days.*

Hybrid	Boron (μM)	SDW (g)	RDW (g)	TRL (cm)	RLR (cm g^{-1})	RMR (g g^{-1})	F (cm cm^{-3})	TD (g cm^{-3})
Ikram	*25*	0.036 a *(0.009)*	0.99 ab *(0.01)*	1874 a *(152)*	1725 a *(83)*	0.036 ab *(0.003)*	3098 a *(122)*	0.055 b *(0.005)*
	320	0.027 a (0.007)	0.72 ab *(0.14)*	1254 b *(187)*	1090 c *(120)*	0.027 b *(0.0007)*	2812 a *(263)*	0.052 b *(0.002)*
	640	0.027 a (0.007)	0.58 ab (0.04)	1371 b (60)	1581 b (80)	0.038 ab (0.001)	3011 a (221)	0.064 a (0.003)
	1280	0.034 a (0.005)	0.72 ab (0.05)	1317 b (37)	1857 a (24)	0.045 a (0.003)	2842 a (60)	0.067 a (0.004)
Losna	*25*	0.031 a (0.007)	0.89 ab (0.05)	1569 ab (30)	1506 b (142)	0.031 b (0.0007)	3166 a (217)	0.053 b (0.002)
	320	0.027 a (0.003)	1.01 ab (0.02)	1711 ab (26)	1454 b (69)	0.027 b (0.0008)	3385 a (519)	0.053 b (0.0009)
	640	0.036 a (0.002)	1.02 a (0.04)	1875 a (58)	1740 a (32)	0.033 ab (0.0008)	2866 a (111)	0.054 b (0.002)
	1280	0.022 a (0.005)	0.42 b (0.02)	1570 ab (101)	1790 a (25)	0.036 ab (0.0009)	3234 a (451)	0.055 b (0.0008)

SDW, Shoot Dry Weight; RDW, Root Dry Weight, TRL, Total Root Length; RLR, Root Length Ratio; RMR, Root Mass Ratio, F, Fineness; TD, Tissue Density). Different letters along the column indicates significant differences (P<0,05, ANOVA, Tukey's test , n=5)

In this work, root length ratio (RLR) and its morphological components, biomass allocation (root mass ratio, RMR) and structural parameters (root fineness and root tissue density, RF and RTD, respectively) were considered as useful root traits of tomato response to B excess. After 7 d of treatment, B differentially affected RLR in two tomato genotypes. Under B excess (640 and 1280 μM), RLR values were significantly increased in Losna by 10 % respect to Ikram associated with a lower

RTD component (-15 %) while both biomass allocation and root fineness parameters were left unchanged (Table 4). Conversely, Ikram showed a variable response of RLR parameter, which however was not significantly changed at highest concentration. Furthermore, in Ikram, the other root morphological components, RMR, F and TD, were not affected by B excess (Table 4). Furthermore, root and shoot dry weights were not affected in both tomato genotypes, while a significant decrease in chlorophyll content was observed already at 640 μM in sensitive Ikram (Figure 6).

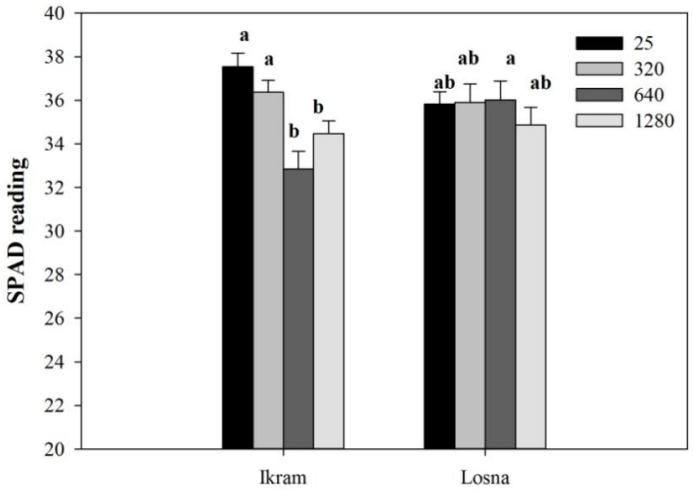


Figura 6. *SPAD reading of two tomato genotypes exposed to 25, 320, 640 or 1280 μM B for 7 days.*

After a short term treatment, B differentially affected shoot and root growth rate of tomato genotypes (Table 4). In particular, shoot growth rate (g dry weight day^{-1}, SGR) was already reduced at 320 μM B treatment (-18% compared to control) in Ikram, and this decrease was also maintained at 640 μM B (Table 5). On the other hand, in Losna, SGR was inhibited at the highest B concentration (640 μM) by 11% compared to control (Table 5). Moreover, the root growth rate (RGR) displayed a similar trend, although more marked, in both genotypes (Table 4). Indeed, the RGR inhibition was statistically significant in Ikram at 320 and 640 μM boron (70 and 65% compared to control, respectively), whereas Losna showed a significant inhibition only at highest B level (50% compared to the control) (Table 5).

However, the effect of B excess on root morphology was evident in both genotypes only after 48 h of treatments, thus the results were referred to this experimental period.

Total root length did not show any significant difference up to 320 µM B treatment in both genotypes, whereas, at highest B level, a significant decrease of TRL was observed in Ikram but not in Losna (Table 5). Moreover, morphological analysis displayed an increase of the RLR parameter in Losna along with increasing boron level (Table 5), accompanied by a root tissue density reduction but without changing in root mass ratio and root fineness as already observed in long-term B treatment (Table 5). In contrast, B excess did not modify all these root morphological parameters in Ikram genotype (Table 5).

Table 5. *Morphological parameters of two tomato hybrids exposed to different boron level for 48 h.*

Hybrid	Boron (µM)	SGR (g DW d^{-1})	RGR (g DW d^{-1})	TRL (cm)	RLR (cm g^{-1})	RMR (g g^{-1})	F (cm cm^{-3})	TD (g cm^{-3})
Ikram	25	0.0011a	0.0002a	594a	4606cd	0.078a	2161a	0.038ab
	320	0.0009b	0.00006c	439ab	4216d	0.083a	1865a	0.037ab
	640	0.0009b	0.00007c	324b	4528cd	0.091a	2225a	0.043d
Losna	25	0.0009b	0.0002a	521ab	5756ab	0.089a	1912a	0.029abc
	320	0.0010b	0.0002a	521ab	5431bc	0.072a	2011a	0.026bc
	640	0.0008c	0.0001b	468ab	6762a	0.071a	1681a	0.018c

SGR, Shoot Growth Rate; RGR, Root Growth Rate, TRL, Total Root Length; RLR, Root Length Ratio; RMR, Root Mass Ratio, F, Fineness; TD, Tissue Density). Different letters along the column indicates significant differences ($P<0,05$, ANOVA, Tukey's test , n=5)

2.2.2 BORON CONTENT

Root and shoot concentrations of B rapidly increased with the toxic treatments in both tomato genotypes exposed to B for 7 d. However, in addition to the treatment, genotype also had a significant effect on the levels of B in both root and shoot. Indeed, Ikram registered the highest root and shoot concentrations of this element at each B level (Figure 7).

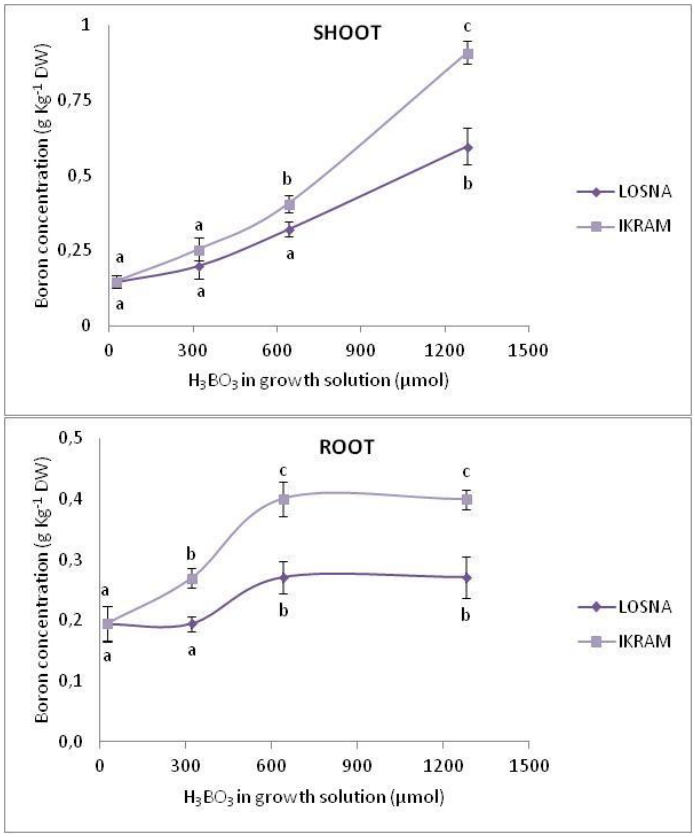


Figure 7. *Boron concentration in shoot and root of two tomato genotypes exposed to different boron concentrations for 7 days.*

2.2.3 NET NITRATE UPTAKE

Significant differences were evident between the two tomato genotypes after exposure to nitrate with or without different B concentrations (Figure 8). In particular, in Ikram seedlings exposed to 25 µM B (control) and 200 µM nitrate, net nitrate uptake occurred immediately (induction phase) and progressively reached a peak of maximum activity after 8 h (full induction) (Figure 8A). Thereafter, a following decline (decay phase) of net nitrate uptake was observed (Time F=25,784***) (Figure 8A). Both B treatments (320 and 640 µM) caused, in Ikram, a considerable decrease on net nitrate uptake during both the induction and full

induction phases compared to the control plants (Boron F= 5,334**). No significant difference between control and B treated seedlings was observed during the decay phase (Figure 8A). Conversely, Losna seedlings, regardless B treatments, exhibited a faster induction phase of net nitrate uptake than Ikram, which was achieved after 4 h of exposure to nitrate (Figure 8B). Furthermore, all B treatments did not significantly affect net nitrate uptake pattern (Boron F= 0,328^{ns}) (Figure 8B).

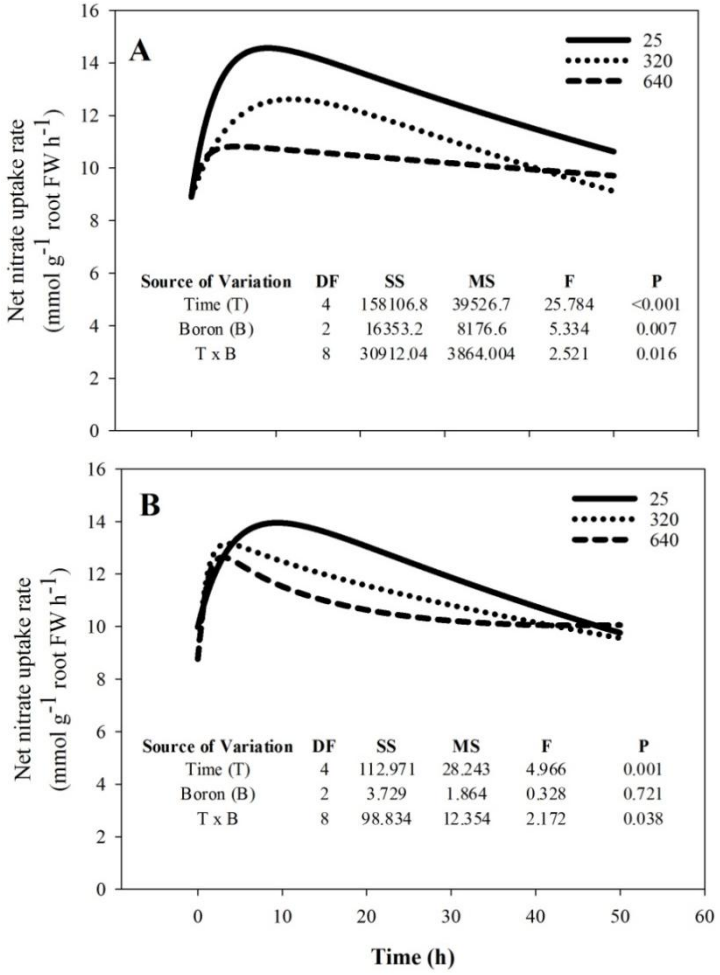


Figure 8. *Time-course of net nitrate uptake rates (μmol NO_3^- hr^{-1} g^{-1} FW) in tomato hybrids (Ikram, A; Losna, B) exposed for 0, 4, 8, 24 and 48 hours to 200 μM nitrate and different boron concentrations. The table indicates the analysis of the variance (n=12).*

2.2.4 PMH+-ATPASE ACTIVITY

Root plasma membrane vesicles results revealed a similar trend between net nitrate uptake and ATP hydrolyzing activity (Figure 9). In particular, a significant reduction in pmH+-ATPase activity in vesicles isolated from Ikram seedlings treated with both B toxic levels for 8 and 24 h was observed compared to control (Figure 9a). In Losna seedlings, pmH+-ATPase activity was not significantly affected by 320 μM B level but its activity tended to decline after 8 and 24 h at highest B treatment (Figure 9b).

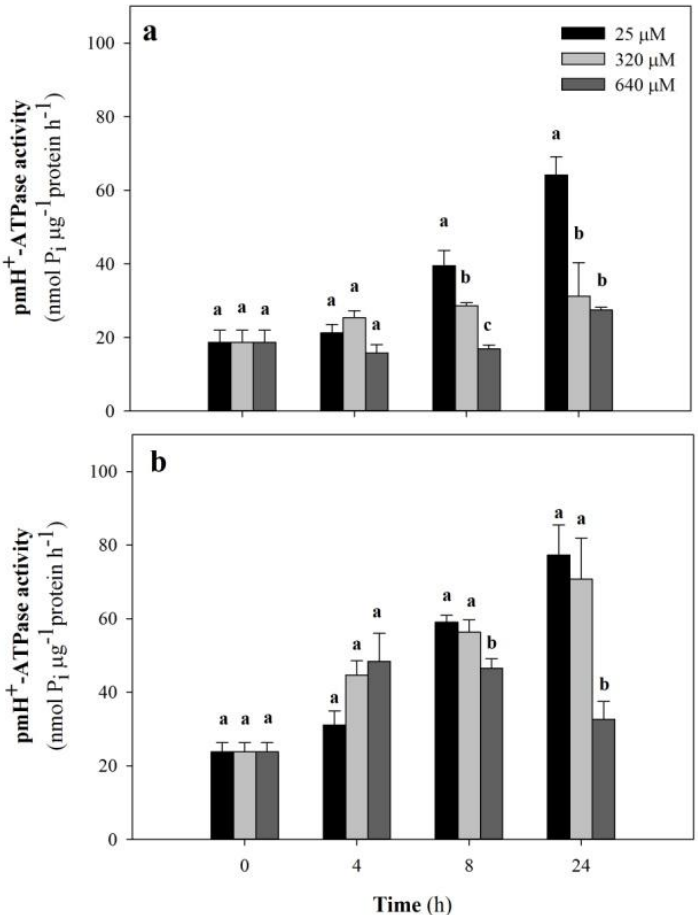


Figure 9. *H+-ATPase activity (nmol Pi μg-1 prot. hr-1) of plasma membrane vesicles isolated from root tomato hybrids (Ikram, A; Losna, B) exposed to 200 μM nitrate and different B concentrations for 0, 4, 8 and 24 hours. Data are the mean of three replicates and and bars indicate the standard error. Within each time of exposure, different letters indicated difference at P < 0.05 (ANOVA, Tukey's test).*

2.2.5 MEMBRANE POTENTIAL MEASUREMENTS

Membrane potential did not significantly vary between tomato root genotypes and this pattern was maintained also after treatment with 2 mM vanadate. After 10 μM coumarin, an activator (inducer stimulator) of pmH$^+$-ATPase, root membrane potential was significantly increased (17%) compared to both roots grown in N-free solution and in vanadate. However, no differences were observed between tomato genotypes in presence of coumarin (Figure 10). Membrane potential recorded after 320 μM B and 200 μM nitrate treatment was instead significantly different between tomato genotypes, displaying a fast membrane hyperpolarization (more negative electrical potential) in Losna compared to Ikram (-21 mV *vs.* -10 mV), which was observed also in presence of B alone (-16 mV *vs.* -7 mV) and B plus coumarin (-18 mV *vs.* -3 mV). On the other hand, after vanadate plus B treatment, Losna showed a lower membrane potential hyperpolarization compared the other treatments, but this effect completely disappeared in Ikram (Figure 11).

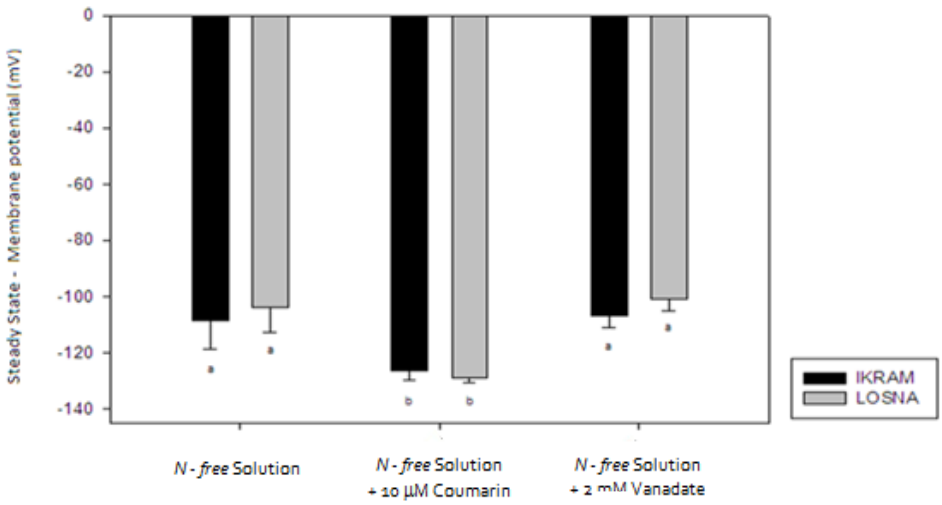


Figure 10. *Membrane potential at steady-state in primary root cells of two tomato hybrids different treated. Different letters indicates means that differ significantly, according to Tukey's HSD test P≤0.05*

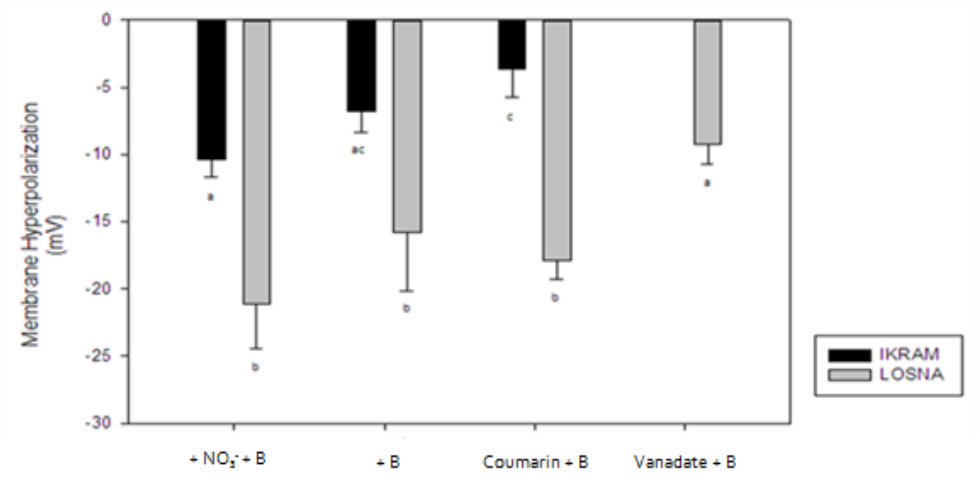


Figure 11*. Membrane hyperpolarization after 30 min of exposure to 200 μM nitrate and 320μM Boron (+NO_3^- +B), or 320 μM Boron (+B), or 320 μM Boron and 10μM Coumarin (Coumarin +B), or 320 μM Boron and 2mM Vanadate of two tomato genotypes. Different letters indicates means that differ significantly, according to Tukey's HSD test P≤0.05*

2.2.6 GENE EXPRESSION ANALYSIS

The expression pattern of *NTR2.1/NAR2.,* the two component of HATS, in tomato root genotypes exposed to 200 μM nitrate with or without different B concentrations (25, 320, 640 μM) were investigated by RT-PCR and qPCR. At time 0 before nutrients supply, the RT-PCR revealed a comparable expression level of *NRT2.1* between hybrids (7.9 and 7.8 were the ΔCt values measured in Losna and Ikram, respectively). After 4 and 8 h of nitrate supply (and 25 μM B) Losna showed a significantly higher amount of mRNA compared to Ikram, especially at 8 h (Figure 12A). *NAR2.1* mRNA abundance appeared similar in both genotypes before treatments (ΔCt 0.7 and 0.8 in Losna and Ikram, respectively), in qPCR this trend was confirmed after 4 h from treatments, while at 8 h Losna showed higher level of *NAR2.1* expression (Figure 12A). The couple genes, *NTR2.1/NAR2.1*, expression level was significantly different between Ikram and Losna after exposure to 200 μM NO_3^- together with 25 (control), 320 and 640 μM B (Figure 13A-B). The effect of B-treatments on gene expression was particularly evident after 4 h, where at both B toxic concentrations *NTR2.1* showed a significantly higher expression in Losna compared to Ikram; in contrast, at 8 h Ikram showed a higher *NTR2.1* expression at

both B concentrations (Figure 13A). *NAR2.1* showed a similar expression pattern considering both hybrids and B concentrations (Figure 13B). It is noteworthy that the addition of B amounts (320 and 640 μM) to a limited concentration of nitrate (200 μM) triggered the increase of genes expression only in Losna after 4 h of treatments compared to the transcripts abundance in the control (25 μM) (Figure 13B). Overall *NRT2.1*/*NAR2.1* expression were strongly inhibited in Losna at 8 h (decay phase), while Ikram at the same time showed a very limited peak of expression (induction phase) compared to the control (Figure 13A-B).

The pmH^+-ATPase genes LHA1, LHA7 and LHA8 in response to nitrate and different B concentrations provision in Ikram and Losna hybrids were investigated by RT-PCR and qPCR. *LHA7* failed to reveal any expression signal in all treatments in our experimental conditions (data not shown). At time 0, gene expression analysis revealed very significant differences in *LHA1* and *LHA8* mRNA abundance between hybrids, where Losna showed for both genes higher ΔCt values compared to Ikram (data not shown). The effect of nitrate were measured by qPCR in the treatments where only 25 μM of H_3BO_3 was added, showing that both *LHA1* and *LHA8* were significantly higher expressed in Losna at both 4 and 8 h after treatments, when compared to Ikram (Figure 12B). The involvement of both genes, with particular emphasis for *LHA8*, in response to nitrate supply is clearly evident in Losna, on the contrary the expression of both genes appeared repressed in Ikram (Figure 12B). The effects of B treatments showed that only Ikram provided with 320 μM B at 4 h showed a limited increase in *LHA1* mRNA abundances compared to the control (Figure 13C). More interestingly, *LHA8* increased only in Losna after 4 and 8 h of B contact and the highest B concentration (640 μM) triggered the highest gene expression, while in Ikram *LHA8* showed a similar behavior of *LHA1* and *LHA7* (Figure 13D).

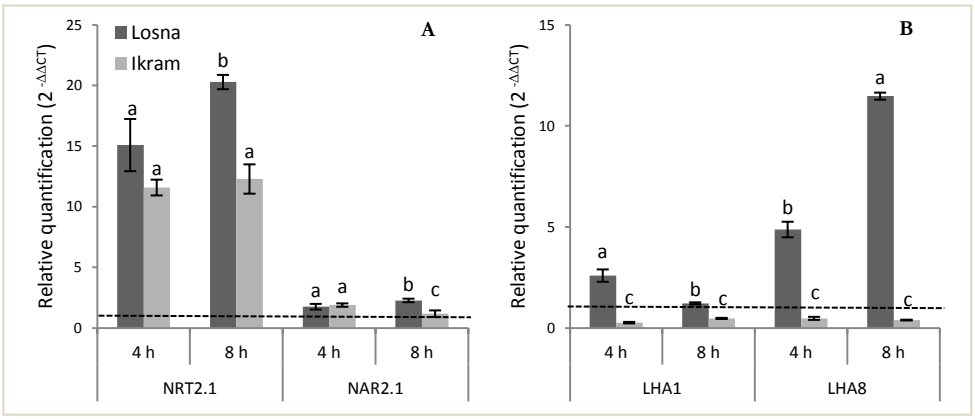


Figure 12. *Gene expression pattern of nitrate transporter family NRT2.1, NAR2.1 and H+-ATPase isoforms LHA1, LHA8 in two tomato hybrids after 4 and 8 h of nitrate (200 μM) supply. Different letter within each gene considered indicate means that differ significantly, according to Tukey's HSD test at P ≤ 0.05.*

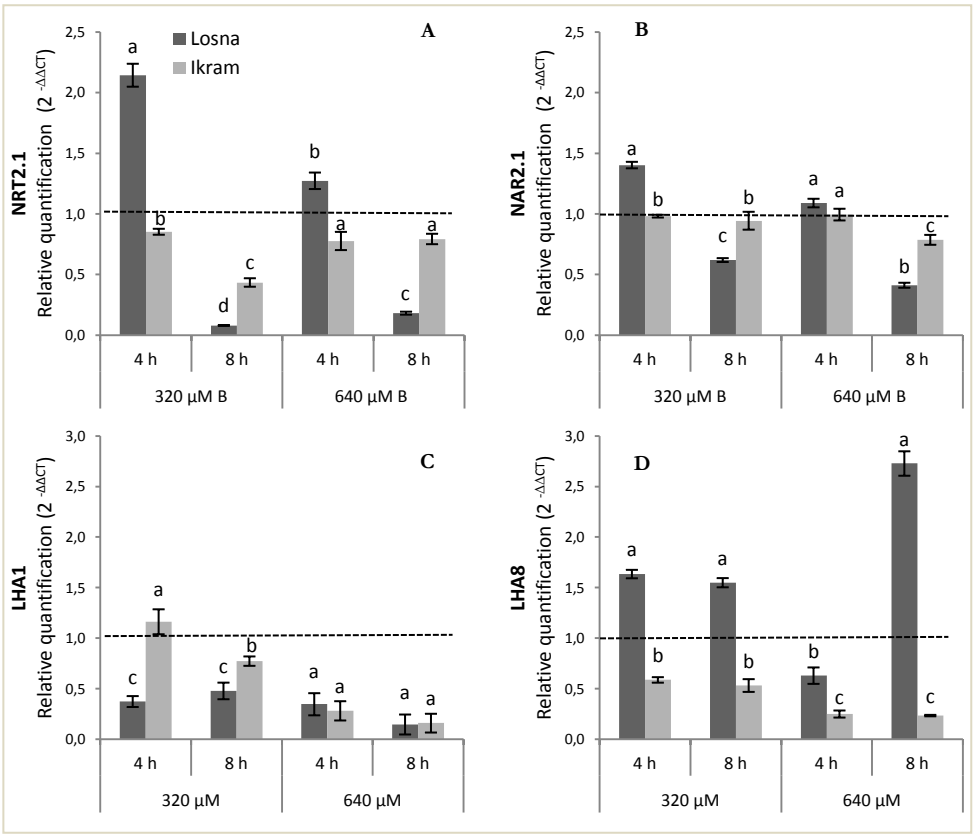


Figure 13. *Gene expression pattern of nitrate transporter family (NRT2.1, NAR2.1) and H+ATPase isoforms (LHA1, LHA8) in two tomato hybrids after 4 and 8 h of B (320, 640 μM) supply. Different letter within each B treatmentt indicate means that differ significantly, according to Tukey's HSD test at P ≤ 0.05.*

Finally, the gene expression of NIP5;1 and BOR1, both important for an efficient B transport across the plasma membrane under B limitation were then assayed by RT-PCR and qPCR in our experimental conditions (Figure 14 A-C). In addition, *BOR4* (paralogue of *BOR1* in *Arabidopsis*) was tested in spite of its effectiveness to increase B-excess tolerance in different species (Figure 14B). Before nitrate and B supply (time 0) the expression of all B transporters genes were significantly different between hybrids, indeed Ikram showed higher *BOR1*, *NIP5;1* and *BOR4* expression compared to Losna as evidenced by ΔCT values (data not shown).

The expression patterns of *BOR1* and *NIP5;1* showed limited differences between hybrids and a lower amount of transcripts in both B-excess concentration conditions compared to the control, as expected (Figure 14 A-C). More interestingly, *BOR4* expression showed significantly differences between hybrids (Figure 14B). In particular, after 4 h of B-treatment Losna showed a higher *BOR4* expression at both 320 and 640 μM H_3BO_3 when compared to Ikram, at 8 h *BOR4* is highly expressed also in Ikram at 320 μM H_3BO_3 while at the highest B concentration Ikram showed higher mRNA abundance then Losna (Figure 14B).

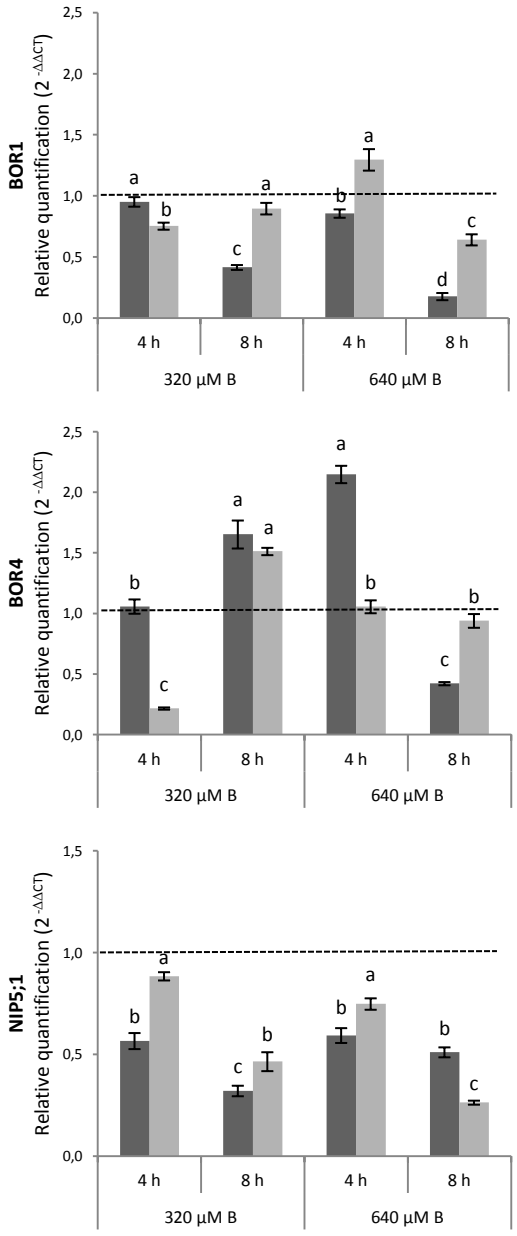


Figure 14. *Gene expression pattern of boron transporter family (BOR1, BOR4) and aquaporin B -channels (NIP5;1) in two tomato hybrids after 4 and 8 h of B (320, 640 µM) supply. Different letter within each B treatment indicate means that differ significantly, according to Tukey's HSD test at P ≤ 0.05.*

2.3 DISCUSSION

Long and short-term boron responses in two contrasting tomato genotypes and its interaction with nitrate, fundamental nutrient for plant growth and development (Crawford, 1995), have been here carried out. Phenotypic assay for B toxicity tolerance between Ikram and Losna genotypes under controlled conditions have been developed, including shoot and root dry weights, root morphological analysis, leaf symptom expression and boron content (Nable, 1988; Campbell *et al.*, 1998; Schnurbusch *et al.*, 2008, 2010). After 7 days of treatment (long-term treatment), Ikram and Losna tomato genotypes showed a different tolerance to high B in culture solution experiments, being Ikram more sensitive than Losna. Indeed, Ikram showed evident leaf symptom (Figure 15) confirmed by reduced chlorophyll content at high B concentrations in both root and shoot compared to Losna.

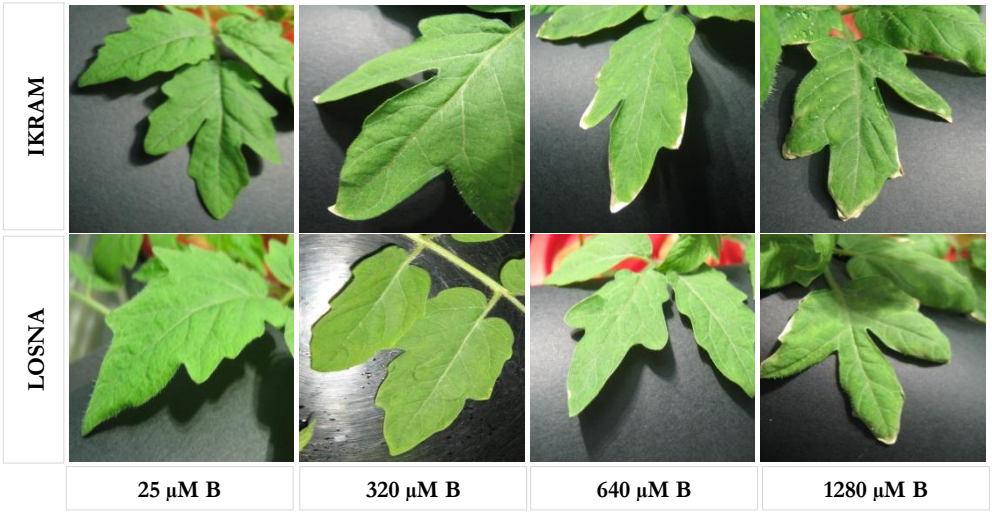


***Figure 15**. Boron toxicity symptoms on leaves of tomato hybrids (Losna, Ikram) at 7 days of exposure to different boron levels (25; 320, 640 and 1280 µM).*

Furthermore, a typical pattern of B toxicity on root growth of two contrasting tomato genotypes was observed. Chantchume *et al.* (1995) found that root length could be used as morphological marker to select for tolerance among wheat genotypes under B toxicity. Indeed, root elongation of sensitive Ikram was immediately and more reduced as external B concentrations increased than tolerant Losna. Different root strategies seemed to be employed by barley cultivars in response to B excess including branching increase, finer roots development and alteration of roots distribution between top- and subsoil (Choi *et al.*, 2006). Thus, a

deeper analysis of root morphological traits between tomato genotypes was performed. The RLR parameter, which indicates how much the plant biomass is used for the development of a more efficient root system especially under stress conditions (Ryser and Lambers, 1995), appeared to be similar between two genotypes under moderate B level (320 μM), rising in response to increasing B concentrations in tolerant Losna. The results suggested that Losna, under B excess, spend more resources in developing a more efficient root system, limiting the effects of high B on the shoot. This may explain both the significant increase in root length and the absence of significant variations of shoot dry weight under B excess. Changes of RLR depend on RMR, RF and RTD components by the following relationship: RLR=RMR×RF/TD (Ryser and Lambers, 1995). Therefore, plants may produce longer roots either by increasing biomass allocation, as demonstrated under low nitrogen supply (Ryser and Lambers, 1995; Sorgonà *et al.*, 2005) or by increasing root fineness and/or reducing root tissue density, leaving biomass allocation unchanged (Ryser, 1998). Such adjustments, defined as root morphological plasticity, allow plants to adapt to uneven distribution of soil resources (Sultan, 2000). The results indicated that B excess did not modify biomass allocation (RMR) and root fineness (RF) in both genotypes, but it was able to increase root tissue density (RTD), an adaptive trait positively correlated with the degree of lignification and cell wall thickness (Hummel *et al.*, 2007). So that, it was possible hypothesized that root growth reduction in Ikram could be caused by an increase in cell wall lignification which in turn reduced cell wall extensibility as demonstrated by Shopfer *et al.* (2001). An adverse B effect on root lignification was already reported in tomato by Cervilla *et al.* (2009). However, this phenomenon has not been generally considered as an essential cause of root growth inhibition, but as a part of the defense reaction which reduced B uptake in soybean and tobacco seedling grown under B excess (Ghanati *et al.*, 2005; 2007).

Since tolerance to B toxicity appeared to be correlated with lower B levels in roots and shoots (Hayes and Reid, 2004), analysis of tissue B concentrations in both genotypes was determined. Shoot and root B concentrations increased along with the toxicity treatments and this effect was different between two tomato genotypes. Ikram showed always a higher B level compared to both the control (25 μM) and Losna and this pattern was evident especially in root. According to Nable (1988), tolerant cultivars maintained lower B concentrations in roots and shoots than sensitive genotypes. Furthermore, according to Hayes and Reid in barley cultivars (2004), these results suggested that the roots of Losna genotype could be able to exclude or efflux B resulting in less accumulation of B in the shoots, important basis for B tolerance.

Early changes (short-term treatment) to B excess on root form and function correlated with several biochemical and molecular aspects of nitrate uptake were also analyzed. Root system appeared to be affected before the occurrence of leaf toxicity symptoms commonly caused by boron excess (Nable *et al.*, 1997; Bennett *et al.*, 1999; Reid *et al.*, 2004; Paul *et al.*, 1992a), as previously observed in barley (Karabal *et al.*, 2003), soybean (Ghanati *et al.*, 2005), cotton (Ahmed *et al.*, 2008) and tomato (Cervilla *et al.*, 2009). These results underlined the high sensitivity of root system to B toxicity which was also confirmed by comparison of RGR with SGR values, especially at lower B level. Similar responses were observed by Cervilla *et al.* (2009a) in tomato and in other species (Kaya *et al.*, 2009; Simòn *et al.*, 2013) but also reported under boron deficiency (Camacho-Cristobal and Gonzales-Fontes, 1999). Overall, root morphology response to short- term B excess indicated that the strategy adopted by the two genotypes was similar to that observed in long- term B treatment on which Losna invested more than Ikram to develop a thinner and longer root system in response to B excess.

It is known that changes in root structure and distribution are usually accompanied by changes in mineral nutrition (Clarkson, 1996), so B excess could differently affect net nitrate uptake pattern between two tomato genotypes. Physiological studies have suggested that at low NO_3^- concentrations, such as the 200 μM adopted in the present research, the HATS significantly contributed to anion uptake (Forde and Clarkson 1999; Forde 2000; Glass *et al.*, 2002; Glass, 2009). The HATS is constituted by two components under different genetic control, a constitutive (cHATS) and an inducible (iHATS), and it is highly regulated in several plant species. In particular, the iHATS is induced by external NO_3^- in nitrate-starved roots, and down-regulated by downstream N metabolites, while cHATS is constitutively expressed (Forde & Clarkson 1999; Forde 2000; Glass *et al.* 2002; Glass, 2009). Regardless of B treatment, Losna and Ikram showed a net nitrate uptake regulation similar to that reported in other species (Tischner, 2000) characterized by an immediate increase of NO_3^- uptake (induction phase), a complete induction (full induction) and a subsequent inhibition of absorption (decay phase), after prolonged NO_3^- contact. Under B excess, a significant inhibitory effect on net nitrate uptake rate (NNUR) was observed in Ikram already at lower B concentration but not in Losna. These results showed for the first time that B excess negatively affected net nitrate uptake rate (NNUR), but this effect was different in relation to B-tolerance of genotypes. Previous studies proved that B toxicity caused inhibition of NO_3^- reduction, increasing NH_4^+ assimilation in tomato (Cervilla *et al.*, 2009a), sunflower (Kastori *et al.*, 1989), barley and wheat (Mahboobi *et al.*, 2002). Interestingly, during the short-term boron deficiency, an inhibition of nitrate uptake

was observed in tobacco roots and it could be responsible of lower leaf and root nitrate contents (Camacio-Cristobal and Gonzales-Fontes 2007).

At molecular level, a NRT2 transporter family potentially encodes HATS components has been isolated in several plants including *Arabidopsis* (Huang *et al.*, 1999), barley (Vidmar *et al.*, 2000), rice (Lin *et al.*, 2000), *Nicotiana plumbaginifolia* (Frasier *et al.*, 2000) and also in tomato (Ono *et al.*, 2000; Wang *et al.*, 2001). A strong correlation among the regulation pattern of iHATS for NO_3^- uptake, *NRT2.1* expression and its functional partner *NAR2.1* has been shown in whole and along root system (Tong *et al.*, 2005; Okamoto *et al.*, 2006; Orsel *et al.* 2006; Wirth *et al.* 2007; Sorgonà *et al.*, 2011). Regardless of boron and consistent with these studies, the present results confirmed the close relationship between iHATS activity and *NRT2.1*/*NAR2.1* expression, and the decisive role played by the *NRT2* family in the induction of NO_3^- uptake in both tomato genotypes. Under B excess, this close correlation between NNUR activity and *NRT2.1*/*NAR2.1* transcript levels was clearly observed in Ikram where a decline of NNUR activity was always accompanied by a drop of *NRT2./NAR2.1* expression. Nevertheless, in Losna, this correlation was evident only at 4 h (full induction), where a higher nitrate uptake mirrored high *NRT2./NAR2.1* transcript abundance also under toxic B level. However, this correlation between nitrate uptake activity and *NRT2./NAR2.1* was lost at 8 h where a marked lower transcript level was observed. At first glance, this result could be explained by an increase of pmH^+-ATPase which could lead to a increase of nitrate transport across the plasma membrane. It is well known that nitrate uptake, as an energy-dependent process, need a favorable electrochemical gradient across the plasma membrane, which is provided by pmH^+-ATPase activity (Miller and Smith, 1996). It has been demonstrated that, in maize roots, pmH^+-ATPase showed a similar time-course pattern to NNUR at biochemical and molecular level (Santi *et al.*, 1995, 2003; Sorgonà *et al.*, 2011). The results confirmed this strong correlation also in both tomato genotypes with or without boron treatment. In Ikram, a drop in pmH^+-ATPase activity and transcript level was associated with a lower nitrate uptake, under B excess, *viceversa* in Losna higher pmH^+-ATPase, at biochemical and molecular level, could justify the higher NNUR activity under B excess.

The physiological basis for tolerance is primarily to limit B accumulation within the plant. In *Arabidopsis thaliana,* NIP5;1 and BOR1 transporter families involved in an efficient B transport across the plasma membrane were successfully isolated and characterized. In particular, NIP5;1, facilitates B influx into root cells, instead BOR1, an efflux-type borate transport, plays a key role in xylem loading and in B distribution within shoots (Takano *et al.*, 2002; 2006). Their increased expression significantly improved vegetative and reproductive growth of *Arabidopsis thaliana*

under B-deficiency (Uraguchi *et al.*, 2014). Conversely, under B excess, BOR1 is incorporated into endosomes and transported to the vacuole for its degradation, beneficial mechanism for plants to avoid B overaccumulation in shoots (Takano *et al.*, 2005). Consistent with its function, a low amount of *NIP5;1* transcripts expression at all B treatments and in both tomato genotypes was observed. Furthermore, as expected, *BOR1* expression patterns at both 320 and 640 μM B concentrations did not show differences between tomato genotypes and appeared rather down-regulated by B excess compared to control (25 μM). These results confirmed that the expression of both genes did not improve plant growth in the presence of toxic B levels.

The isolation of *BOR4*, a paralogue of *BOR1* in *Arabidopsis,* responsible for directional B export from the roots to the soil, and its over-expression in transgenic plants successfully increase tolerance to high B (Miwa *et al.*, 2007). Transgenic plants tolerated 10 mM boric acid in the medium, while the untransformed wild-type plants barely extended roots. In contrast to BOR1, BOR4 is not degraded even under high levels of B supply (Miwa *et al.*, 2007). More interestingly, *BOR4* expression showed significantly differences between contrasting tomato genotypes in our experimental conditions. Indeed, after 4 h of B-excess treatment, Losna showed a higher *BOR4* expression at both B concentrations and time of exposure compared to Ikram.

So that, the most important mechanism able to explain the tolerance of Losna is based on boron efflux from roots. Hayes and Reid (2004) proposed two models of tolerance: an anion channel (Model I) and an anion exchange (Model II). In particular, the first model is strongly dependent on borate permeability and needed energy input to actively extrude H^+ (*via* H^+-ATPase) to maintain the electrical driving force for borate efflux. They postulated that B-tolerance mechanism in barley cultivar Sahara was based on the presence of a borate anion efflux transporter able to actively extruded B from the roots and this efflux increased along with increasing H^+ concentration.

An active boron efflux through BOR-type transporters driven by concentrations gradients across plasma membrane and maintained by an energy input was recently postulated (Reid, 2014). Since the driver ion for co-transport is usually H^+ in plants, simple electrophysiological experiments in presence of stimulator or inhibitors of H^+-ATPase were performed in roots of both tomato roots.

The results strongly suggested the involvement of an electrogenic proton efflux in tomato tolerance B mechanism. Indeed, B-tolerant Losna showed a higher membrane potential hyperpolarization than Ikram in response to B. This effect was also evident when tomato roots were simultaneously exposed to B and nitrate or

coumarin, which generally caused a membrane potential hyperpolarization (McClure *et al*., 1990; Lupini *et al*., 2013). Thus, the first hypothesis of B-tolerance in tomato was that borate efflux was driven by electrogenic proton efflux *via* H^+-ATPase. However, it is well known that metabolic inhibitors such as vanadate would strongly inhibit electrogenic H^+ pump and consequently the electrical gradients that drive the B co-trasport reaction. Interestingly, in presence of B and vanadate, Losna, showed a low membrane hyperpolarization yet. What does it mean ? As suggested by Roldan *et al*. (1992) a passive component in the H^+ release from roots or the presence of H^+ transport system at plasma membrane other than from H^+-ATPase such as redox systems. Goldbach *et al*. (1990) demonstrated a stimulatory B effect on the ferricyanide-induced proton efflux in carota and tomato suspension cultured cells after auxin treatment. In this respect further experiments have to be performed to demonstrate this hypothesis.

CHAPTER 3 SHORT-TERM ANTIOXIDANT RESPONSES OF TWO TOMATO ROOT SYSTEMS WITH DIFFERENT SENSITIVITY TO B TOXICITY

3.1 MATERIALS AND METHODS

3.1.1 PLANT MATERIAL AND GROWTH CONDITION

In the present study two tomato hybrids (Losna and Ikram), showing different sensitivity to B toxicity, were used. The growth conditions of the plants were the same previously described in Chapter 2 paragraph 2.1.1.

The B treatments (320 or 640 μM H_3BO_3 in the nutrient solution) were imposed when tomato seedlings were 19 d-old, whereas control plants received the complete nutrient solution alone, containing 25 μM H_3BO_3. Tomato roots were sampled after 0, 4, 8, 24 and 48 hours from the beginning of the B treatments. The roots were rinsed three times in distilled water, blotted with filter paper, immediately frozen in liquid N_2 and then stored at -80 °C.

3.1.2 DETERMINATION OF MALONDIALDEHYDE

Malondialdehyde (MDA) is an adehydic product of the oxidative lipid breakdown whose level in plant tissues is frequently assumed to reflect the extent of membrane lipid peroxidation (Heath and Packer, 1968). Thiobarbituric acid-reactive substances, among which MDA, were extracted by homogenizing 0.25 g frozen root sample in 5 mL of a 0.1% (w/v) solution of trichloroacetic acid. The homogenate was then filtered, centrifuged at 4 °C for 5 min at 11,000 rpm, immediately frozen in liquid N_2 and stored at -80 °C until used. An aliquot of 1 mL of the supernatant was added to 1 mL of 1 % (w/v) 2-thiobarbituric acid **(**4,6-dihydroxy-2-mercaptopyrimidine)**,** incubated in a water bath for 30 min at 90 °C and then quickly transferred into an ice bath to arrest the reaction. The intensity of the developed color was recorded at 532 and 600 nm (UV-Vis Shimadzu 2100, Japan). After subtracting the non-specific absorbance at 600 nm, the MDA concentration was calculated using its molar extinction coefficient of 155 $mmol^{-1}$ cm^{-1} (Dhindsa *et al.*, 1981).

3.1.3 DETERMINATION OF HYDROGEN PEROXIDE

Hydrogen peroxide (H_2O_2) is an uncharged compound which can easily diffuse through membranes and react with transition metal reductants or catalysts, forming reactive radicals. Quantification of H_2O_2 levels in plant material was

achieved spectrophotometrically by the ferric-xylenol orange assay as reported by Jiang *et al.* (1990). The method is based on the peroxide-mediated oxidation of Fe^{+2} to Fe^{+3}, followed by the reaction of Fe^{+3} with xylenol orange (*o*-cresolsulfonephtalein 3'-3'-bis[methylimino]diacetic acid, sodium salt). Frozen root samples (0.20 g) were homogenized with 2 mL of 0.2 M perchloric acid ($HClO_4$), the extract was kept on ice for 5 min and then centrifuged (10 min, 11000 rpm, at 4 °C). The supernatant was collected and processed immediately, thus no substantial auto-oxidation of H_2O_2 was observed. The acidic supernatant was neutralized to pH 7.0–8.0 with 0.2 M ammonium hydroxide (NH_4OH) pH 9.5. The coloured components in the extract were removed by applying the extract to a 2 mL column of AG 1-X8 resin (Bio-Rad Laboratories, Hercules, CA, USA) and elution with 3 mL of double-distilled water, immediately frozen in liquid N_2 and stored at -80 °C until use.

H_2O_2 was determined in duplicate by adding 500 µL of each extract to 500 µL of assay reagent containing 500 µM ammonium ferrous sulphate, 50 mM H_2SO_4, 200 µM xylenol orange and 200 mM sorbitol.

Absorbance of the Fe^{+3}-xylenol orange complex was measured at 560 nm after 45 min of incubation at room temperature, using a UV-Vis spectrophotometer (Perkin-Elmer, Norwalk, CT, USA). The amount of H_2O_2 was calculated using a standard curve in the range 0.5 nM – 2.5 µM H_2O_2, obtained under the same conditions reported above.

3.1.4 ANTIOXIDANT ENZYME ASSAYS

3.1.4.1 Enzymes extraction

For enzyme extraction, frozen root samples were placed in a mortar containing liquid N_2, crushed with a pestle to a fine powder and thereafter homogenized with 4 volumes of ice-cold 50 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7.0, containing 5 mM 2-mercaptoethanol, 2 mM dithiothreitol, 2 mM sodium-hydrogen-EDTA, and 1% (w/v) insoluble polyvinylpyrrolidone (Paolacci *et al.*, 1997). After centrifugation at 12000 rpm for 10 minutes at 4 °C, the supernatant was immediately frozen in liquid N_2 and stored at -80°C until its use for the assay of proteins and enzymes.

3.1.4.2 Peroxidase activity

Hydrogen donor-aspecific peroxidases (POD; EC 1.11.1.7) catalyses the oxidation of a wide variety of organic and inorganic substrates using hydrogen peroxide as the electron acceptor. POD activity was determined spectrophotometrically by measuring the enzyme-catalyzed transformation of

guaiacol to tetraguaiacol (a brown product) in the presence of H_2O_2, as described by Forbusch (1983). In a final volume of 2.993 mL, the assay mixture contained 33 mM 2-methoxyphenol **(**guaiacol) in 50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-Cl buffer pH 7.40 and 20 μL of enzyme extract.

The reaction was initiated by adding 7 μL of a 3% (v/v) H_2O_2 aqueous solution. The oxidation of guaiacol was recorded as the increase in absorbance at 470 nm for three minutes and the enzyme activity was calculated using Δ A_{470} min^{-1} chosen in the linear portion of the kinetic plot. One unit of POD activity was arbitrarily defined as being contained in the volume of enzyme extract causing an increase of 1.0 in A_{470} under the reported assay contiditions. POD activity was expressed as units mg^{-1} protein.

3.1.4.3 Superoxide dismutase activity

Superoxide dismutase (SOD; EC 1.15.1.1) acts as an important primary defense against superoxide radical ($\cdot O_2^-$)-mediated cell damage by converting $\cdot O_2^-$ to H_2O_2. SOD activity was determined as described by Elstner *et al.* (1983). Briefly, the method is based on a reference reaction in which the first step is the generation of $\cdot O_2^-$ by a microbial NADH-diaphorase, followed by the $\cdot O_2^-$ -mediated oxidation of hydroxylamine to nitrite. Nitrite is then quantified colorimetrically (A_{540}) following its reaction with sulphanilamide and naphthylethylene diamine. The dismutation of $\cdot O_2^-$ by SOD inhibits the oxidation of hydroxylamine to nitrite, so decreasing the colorimetric yield of the reference reaction in a concentration-dependent manner.

For calculating catalytic activity, a SOD standard curve was built by employing six concentrations of commercially available SOD. One unit of SOD activity was arbitrarily defined as being contained in the volume of enzyme extract causing a 50% inhibition of the reference reaction. Specific activity was expressed as SOD units per mg^{-1} protein

3.1.4.4 Protein assay

Total soluble proteins in roots extracts were determined by the Bradford method (Bradford, 1976). Albumin powder from bovine serum was used as the standard. The Bradford dye assay is based on the equilibrium between three forms of Coomassie Blue G dye. Under strongly acid conditions, the dye is most stable as a doubly-protonated red form. Upon binding to protein, however, it is most stable as an unprotonated, blue form.

For each roots extract, duplicate assays were run by adding 20 μL of sample to 5 mL of an 1:4 (v/v) aqueous dilution of commercial Bradford reagent (Bio-Rad Protein Assay Reagent, Bio-Rad Laboratories), following the manufacturer's instructions. After stirring and incubating at room temperature for 10 to 15 minutes,

the absorbance was read at a wavelength of 595 nm using an UV-Vis spectrometer (Perkin Elmer).

3.1.5 STATISTICAL ANALYSES

The mean and standard deviation (SD) of three replicates were calculated. A parametric three-way analysis of variance (ANOVA), followed by a post-hoc multiple comparison (Tukey's Test), was employed to test any significant differences in response to B toxicity. All statistical analyses were performed with SPSS Version 16.0 (SPSS Inc., Chicago IL, USA).

3.2 RESULTS AND DISCUSSIONS

Superoxide radical anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical and singlet oxygen commonly known as reactive oxygen species, ROS, are considered as an immediate and direct consequence of aerobic life on Earth. Under aerobic condition, cells manage to maintain an adequate balance among ROS formation and scavenging. On the other hand, ROS hyperproduction arises directly or indirectly from a wealth of endogenous or environmental stimuli causing oxidative stress. If not detoxified, ROS accumulate in the cells, causing oxidation of proteins and nucleic acids, as well as membrane lipid peroxidation, with the consequent formation of aldehydic products such as malondialdehyde (MDA) (Heath and Packer, 1968; Mittler, 2002; Del Rio *et al.*, 2003).

Although all ROS could be considered chemically aggressive molecules, properties such as intrinsic reactivity, polarity and electrical charge, make ROS different each. So they can selectively move from the site of formation to their biological target. In this context, H_2O_2 is considered as a particular ROS, because it is less chemically reactive and uncharged, hence it is able to easily cross through biological membranes. The fate of H_2O_2 in plants can be: (i) converted spontaneously or with the participation of catalase to form water and molecular oxygen; (ii) used as a substrate by various hydrogen-donor aspecific peroxidases, e.g. for the generation of phenoxyl radicals, which are building blocks for lignin synthesis (see below): or iii) detoxified by ascorbic acid (AsA) peroxidase which, in the presence of glutathione (GSH) and by acting in concert with four different enzymes, cooperatively bring about H_2O_2 reductive detoxification at the ultimate expense of NAD(P)H (ascorbate-glutathione cycle; Foyer and Noctor, 2005).

Beside being agents of oxidative stress, ROS as certain their oxidative by-products can act as secondary messengers in many processes associated with plant growth and development (Foyer and Noctor, 2005). Moreover, plants can rapidly

and efficiently change information with their surrounding environment via a superoxide/ H_2O_2 burst at the plasma-membrane level (Bolwell *et al.* 2002; Mittler, 2002). Consistently, ROS have been shown to act as essential components in the signal transduction cascade(s), thus leading to defence reactions, such as the programmed cell death and systemic resistance (Foyer and Noctor, 2005).
ROS overproduction in the plant cells requires the activity of antioxidant systems (Foyer and Noctor, 2005), which include metabolites, such as ascorbate (AsA) and reduced glutathione (GSH), and scavenging enzymes, such as superoxide dismutase (SOD), converting superoxide radical anion to H_2O_2, catalase and hydrogen donor-specific peroxidases, such as AsA peroxidases. Instead, hydrogen-donor aspecific peroxidise (POD), which are often found in the apoplastic spaces, is involved in lignification, suberization, cross-linking of hydroxyproline-rich wall proteins and feruloyilated polysaccharides, oxidation and polymerization of soluble phenolics, formation of H_2O_2, senescence, and chlorophyll and auxin degradation.

Boron toxicity symptoms in plants cause leaf chlorotic and/or necrotic patches, particularly at margins and tips of older leaves in species where B is phloem-immobile (Bergmann, 1992), or fruit disorders (gummy nuts and internal necrosis), and bark necrosis in species where B is phloem-mobile (Brown and Hu, 1996). Furthermore, B excess in plants causes reduced root cell division (Liu and Yang, 2000), decreased shoot and root growth, lower stomatal conductance (Lovatt and Bates, 1984; Nable *et al.*, 1997), decrease in leaf chlorophyll, inhibition of photosynthesis, increase in deposition of lignin and suberin (Ghanati *et al.*, 2002), and reduced proton extrusion from roots (Roldan *et al.*, 1992).

Lignosuberization of root cortical cell walls was suggested as a relevant strategy of the plant by which the radial transport of water and B toward conductive tissues is hindered (Ghanati *et al.*, 2005). Then, Choi *et al.* (2007) reported that in barley B tolerance could be associated with root morphological changes and a complex control of sucrose levels between leaf and root tips, which help maintain root growth. This confirms the main role of root system in response to B stress.
Many if not all of the effects of B excess in plants might be due to either direct or indirect consequences of oxidative stress, which, as for many other stresses, has been reported to occur in several plant species. Indeed, ROS accumulation has been reported in apple rootstock (Mollasiotis *et al.*, 2006), wheat (Gunes *et al.*, 2007), barley (Inal *et al.*, 2009) and tomato (Cervilla *et al.*, 2007). Furthermore, B excess induces oxidative damage through lipid peroxidation (Karabal *et al.*, 2003; Keles *et al.*, 2004; Gunes *et al.*, 2006, Molassiotis *et al.*, 2006). Although much information is available on the capacity of B excess to induce oxidative stress in plant tissues, it is still much less clear whether the subsequent activation of plants antioxidant defence systems is

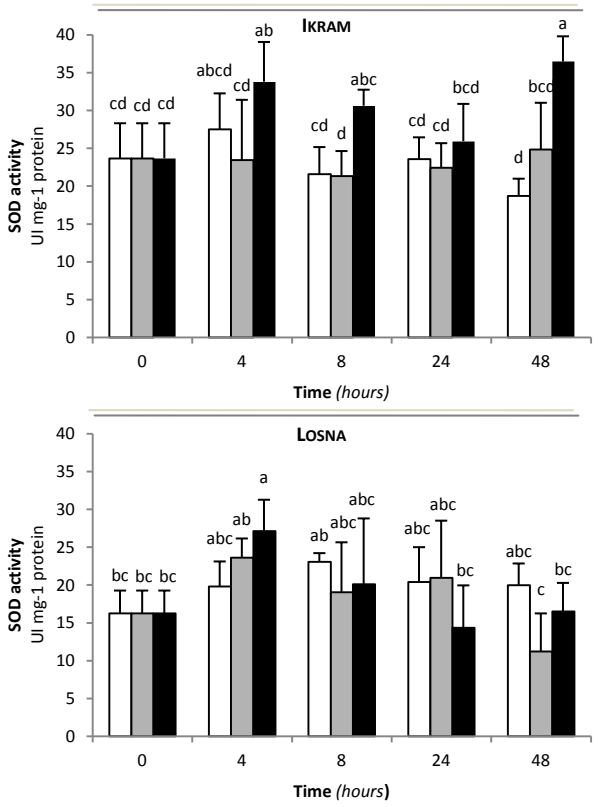
involved in conferring tolerance to B toxicity. Gunes *et al.* (2006), Cervilla *et al.* (2007), and Ardic *et al.* (2009) suggested that an efficient antioxidant response reduces B toxicity damage in grapevine, tomato and chickpea. However, other studies (Karabal *et al.*, 2003; Eraslan *et al.*, 2007; Wang *et al.*, 2011; Hamurcu *et al.*, 2013) reported variable effects on ROS-scavenging enzymes, depending on plant species, cultivars, tissues and B concentrations. This casted some doubt about the direct involvement of antioxidant systems in conferring tolerance to B excess. Because of such wide inherent variability in responses to toxic B levels (Blewins and Lukaszewski, 1998; Kalayici *et al.*, 1998; Cervilla *et al.*, 2007), cultivar, varieties or biotypes belonging to the same plant species but exhibiting contrasting sensitivity to B excess would be a clear advantage to understand B tolerant mechanisms (Karabal *et al.*, 2003). Another open question concerns the tissue- and organ-specific occurrence of B-induced oxidative stress; this is because, while the root is obviously the first organ which perceives B stress, the bulk of B taken up by the root is rapidly translocated to the shoot, along with the transpiration stream (Kalayici *et al.*, 1998; Parks and Edwards, 2005; Cervilla *et al.*, 2009). In this respect, in only one case (Gunes *et al.*, 2006) studying prooxidants/antioxidants in response to B excess has been accompanied by assessing the root-to-shoot distribution of the B supplied to plants.

In the present PhD thesis, several pro-oxidants and antioxidants were selected to investigate any early physiological warning in response to high B levels in Ikram and Losna roots, two tomato genotypes with contrasting B tollerance (Princi *et al.*, 2013; present PhD Thesis). In particular, the main aim was to assess under B excess, the H_2O_2 accumulation and its possible association with MDA levels, the induction of enzymatic ROS scavenging, assuming SOD as a meaningful activity, and, finally, the involvement of lignosuberisation at the root level, taking the activity of a hydrogen donor-aspecific POD as marker.

The results indicate that SOD activity was remarkably higher in Ikram than in Losna roots, and it also appeared to be preferentially stimulated in this roots genotype exposed to the highest B level (640 μM B), although statistical significance was seldom attained. Generally, such stimulation appeared to be biphasic in nature, by peaking after 4 and 48 h after the beginning of the treatment. Conversely, in Losna roots exposed to 640 μM B SOD activity tended to be slightly stimulated only after 4 h of exposure, and then tended to decline. No clear effect on SOD activity was noticed in the roots of both tomato genotypes exposed to 320 μM B (Figure 16).

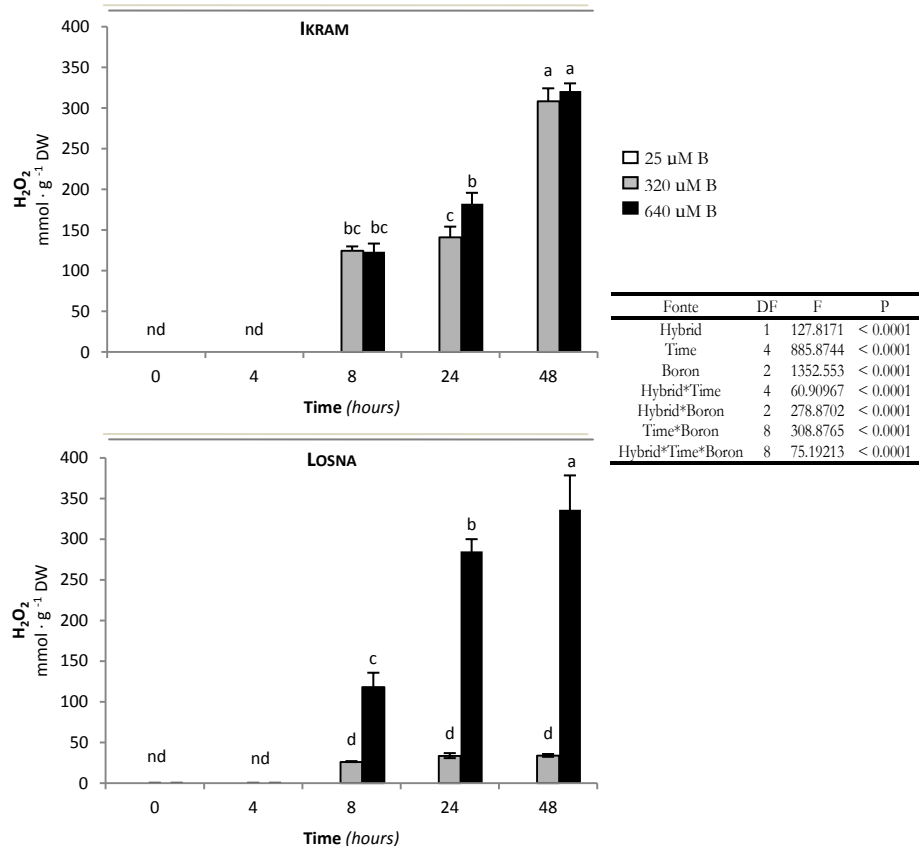
Under moderate B supply (controls), H_2O_2 failed to accumulate over time in the roots of both tomato genotypes. Conversely, under B supply as high as 320 or 640 μM H_2O_2 levels rapidly and progressively started to increase in Ikram roots from 8 h

onwards, whereas in Losna roots this effect became clear only at the highest B concentration. Since H_2O_2 is one of the products arising from SOD catalysis, it is possible to speculate that H_2O_2 accumulation in the tomato roots after 8 h of exposure to B (Figure 17) might be temporarily correlated with the stimulation of SOD activity appearing 4 h before (Figure 16) probably due to an hypothetical accumulation of $\cdot O_2^-$. Such an event chain would be reminiscent of an oxidative burst-type mechanisms, whose occurrence in plants is increasing recognised as an almost universal stress perception/signalling route (Foyer and Noctor, 2005). However, by comparing Figure 16 and Figure 17, it is possible to observe that H_2O_2 accumulation occurred also in the absence of SOD induction in the preceding period, as in the case of Ikram exposed to 320 µM B. Furthermore, no O_2^- accumulation in response to B stress was observed in tomato roots by Cervilla *et al.* (2009), who observed that H_2O_2 accumulated instead, although on a time scale - 4, 8 and 16 days - not comparable to the present one.



Fonte	DF	F	P
Hybrid	1	85.475	< 0.0001
Time	4	8.458	< 0.0001
Boron	2	11.084	< 0.0001
Hybrid*Time	4	3.101	0.017
Hybrid*Boron	2	12.631	< 0.0001
Time*Boron	8	3.404	0.001
Hybrid*Time*Boron	8	3.926	0.000

Figure 16. *Effect of 25 µM B (control) and B toxicity (320 and 640 µM) after 0, 4, 8, 24 and 48 hours of treatments on SOD activity in root of two tomato hybrids: Ikram and Losna. Data are the mean of three replicates and bars indicate the standard error. The table indicates the analysis of three-way analysis of variance (ANOVA,* Tukey's test*) (n=3).*

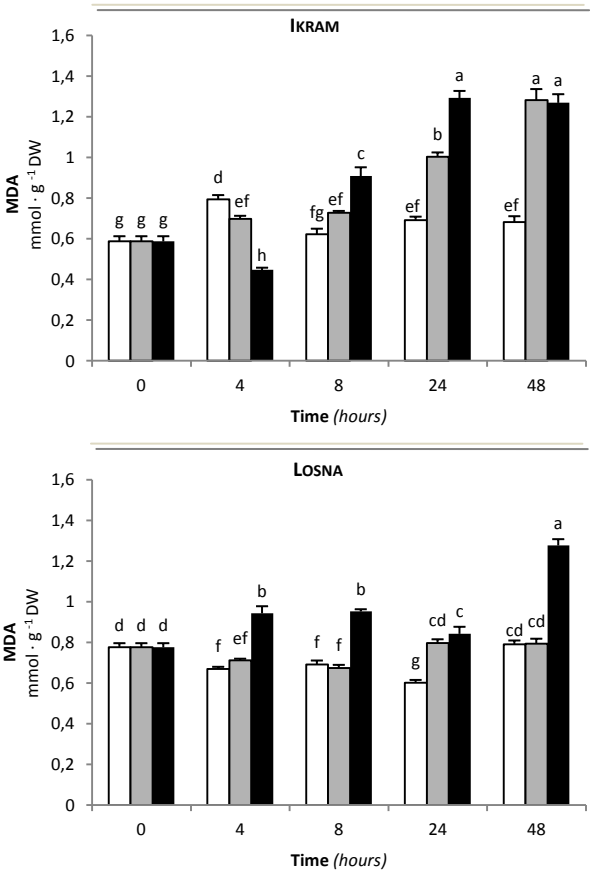


Fonte	DF	F	P
Hybrid	1	127.8171	< 0.0001
Time	4	885.8744	< 0.0001
Boron	2	1352.553	< 0.0001
Hybrid*Time	4	60.90967	< 0.0001
Hybrid*Boron	2	278.8702	< 0.0001
Time*Boron	8	308.8765	< 0.0001
Hybrid*Time*Boron	8	75.19213	< 0.0001

Figure 17. *Effect of 25 µM B (control) and B toxicity (320 and 640 µM) after 0, 4, 8, 24 and 48 hours of treatments on H_2O_2 concentration in root of two tomato hybrids: Ikram and Losna. Data are the mean of three replicates and bars indicate the standard error. The table indicates the analysis of three-way analysis of variance (ANOVA, Tukey's test) (n=3).*

In brief, the source(s) of H_2O_2 accumulation in response to B excess remains to be identified. Similarly, the mechanism(s) preventing the H_2O_2 burst in Losna roots exposed to 320 µM B, but not in Ikram ones, certainly deserves further investigation. In any case, the differences among the early time courses of H_2O_2 accumulation in the seedlings of the two tomato genotypes matched their differential proneness to develop signs and symptoms of B toxicity later on during their development (see the first part of the present PhD Thesis).

Both levels of B excess, and especially the higher one, caused in Ikram roots, MDA accumulation from 8 h onwards. Instead, in Losna roots, 320 μM B did not induce any effect on the MDA content, whereas 640 μM B increased MDA levels starting from 4 h onwards (Figure 18). Therefore, like as for H_2O_2 accumulation, the results of MDA at the early stage of B-induced stress seem to justify and to anticipate the long term differential response exhibited by two tomato genotypes towards B excess.



Fonte	DF	F	P
Hybrid	1	1.520	0.222
Time	4	484.855	< 0.0001
Boron	2	619.954	< 0.0001
Hybrid*Time	4	210.502	< 0.0001
Hybrid*Boron	2	85.914	< 0.0001
Time*Boron	8	147.162	< 0.0001
Hybrid*Time*Boron	8	111.897	< 0.0001

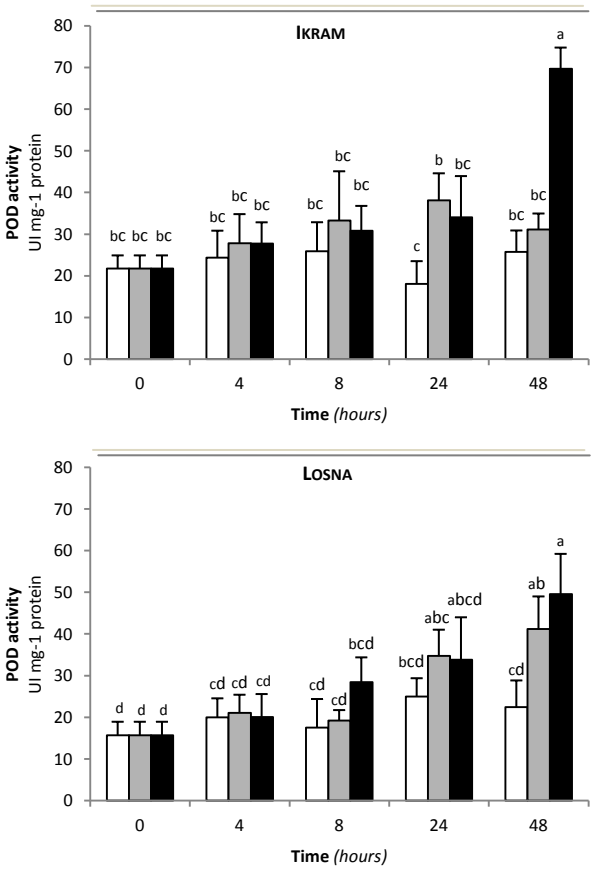
Figure 18. *Effect of 25 μM B (control) and B toxicity (320 and 640 μM) after 0, 4, 8, 24 and 48 hours of treatments on MDA concentration in root of two tomato hybrids: Ikram and Losna. Data are the mean of three replicates and bars indicate the standard error. The table indicates the analysis of three-way analysis of variance (ANOVA, Tukey's test) (n=3).*

Unlike the SOD, POD specific activity (Figure 19) tended to increase in the roots of both tomato genotypes since the early stage of the exposure to B excess. Furthermore this increase was barely detectable after 8 h and became more pronounced after 24 h (Figure 19). Apparently, both levels of B excess were able to stimulate POD activity to the same extent, except in Ikram after 48 h of treatment, where POD more than doubled in response to 640 μM B. As observed with SOD, POD activity was inherently higher in Ikram than in Losna genotype. Although POD response to B excess was prompt and marked showing a root specific activity more than doubled in both genotypes within the first 48 h of B exposure. Furthermore it was by 40% higher in the B-sensitive Ikram than in the moderately tolerant Losna after 48 h (Figure 19), and no remarkable differences were noticed among the time profiles of the two tomato genotypes. Thus, no definitive conclusion can be drawn on a direct contribution of this enzyme in the expression of genotypic tolerance. Likewise, no clear relationship can be state at this stage between B-induced POD activity and root morphological traits, such as increased root tissue density, resulting in lignification and increased cell wall thickness in Ikram roots (Hummel *et al.*, 2007). Furthermore, POD is known as "general stress enzyme" and its induction under B excess was strictly related to the accumulation of its oxidising substrate, namely H_2O_2 (compare Figure 19 and Figure 17). This finding suggests that POD could be involved in removing H_2O_2 excess and could act as a cross-linking agent in lignosuberization, a process that has been previously reported to occur in the root cortex in response to B excess (Cervilla *et al.*, 2009).

The results reported are fairly contrasting with those stated in literature because of a) the majority of the studies on the plant antioxidant status affected by B excess were limited on plant aerial tissues such as leaves and stems (e.g. Gunes *et al.*, 2006; Molassiotis *et al.*, 2006; Cervilla *et al.*, 2007; Eraslan *et al.*, 2007; Aftab *et al.*, 2010; Wang *et al.*, 2011; Hamurcu *et al.*, 2013); b) the very early stages of the interaction between B excess and plant tissues were not adequately considered in other studies; c) B levels much higher than those tested here were generally applied to elicit antioxidant responses; d) comparative responses to B excess using two genotypes showing a contrasting B sensitivity have been rarely investigated (Karabal *et al.*, 2003; Hayes and Read, 2005).

Evidences of straightforward H_2O_2 accumulation and then MDA increase in response to B excess, often assuming H_2O_2 as the causative agent MDA, were observed in leaves of both herbaceous and wooden species: precisely tomato (Cervilla *et al.*, 2007; 2009), Indian mustard (Pandey *et al.*, 2013), apple tree (Molassiotis *et al.*, 2006), Asian pear tree (Wang *et al.*, 2011), and Indian mustard roots (Pandey *et al.*, 2013). It is also noteworthy that there are several studies

reporting on: a) an "uncoupled" response of H_2O_2 and MDA to B excess, as observed in lettuce (Eraslan *et al.*, 2007), grapevine (Gunes *et al.*, 2006), and barley (Karabal *et al.*, 2003); b) non-linear correlation between H_2O_2/MDA accumulation and B excess, as occurred in sweet wormwood (*Artemisia annua* L.; Aftab *et al.*, 2010) and soybean (Hamurcu *et al.*, 2013) MDA accumulation occurred in association with several stress traits, such as drought sensitivity, not directly related to B sensitivity, as observed in chickpea roots (Ardic *et al.*, 2009).



Fonte	DF	F	P
Hybrid	1	13.338	0.001
Time	4	30.088	< 0.0001
Boron	2	25.804	< 0.0001
Hybrid*Time	4	1.483	0.219
Hybrid*Boron	2	0.945	0.394
Time*Boron	8	9.239	< 0.0001
Hybrid*Time*Boron	8	2.581	0.017

Figure 19. *Effect of 25 µM B (control) and B toxicity (320 and 640 µM) after 0, 4, 8, 24 and 48 hours of treatments on POD activity in root of two tomato hybrids: Ikram and Losna. Data are the mean of three replicates and bars indicate the standard error. The table indicates the analysis of three-way analysis of variance (ANOVA, Tukey's test) (n=3).*

Moreover, SOD and POD activities in response to B excess are not univocal in the available literature. Several reports indicate a direct relationships between these enzymatic activities in leaves and roots with the increasing B levels in the growth medium (Molassiotis *et al.*, 2006; Cervilla *et al.*, 2007, 2009; Eraslan *et al.*, 2007; Ardic *et al.*, 2009; Aftab *et al.*, 2010; Pandey *et al.*, 2013), on the other hand other studies described a high variability of SOD and POD activities in response to B increase (Gunes *et al*, 2006; Wang *et al.* 2011; Hamurcu *et al.*, 2013). Indeed, Karabal *et al.* (2003) used B levels from 8 to 16 times as the highest dosage used here (640 μM B) and found no clear effects of B excess on the extractable activities of SOD and other ROS-scavenging enzymes. This finding may lead to the conclusion that "….antioxidant enzyme activity is not a critical factor in boron toxicity tolerance mechanism".

If POD activity was more properly considered as a component of the multi-enzymatic complex driving the oxidative polymerisation of lignin precursors instead of a "mere" ROS-scavenging enzyme, its response to increasing B excess was in accordance with its role played in cell wall lignification of tomato root cortex (Cervilla *et al.*, 2009), although such responsiveness was found to vary with the genotypes studied.

Despite the limited data, the results reported here constitute the first attempt to explore the early events in response to B stress affecting the root antioxidant status in two tomato genotypes that show contrasting B sensitivity. Provided the individual components of the pro-oxidant/antioxidant balance are to be studied more extensively, such an approach would allow to not only to find out the causes and consequences of oxidative stress and damage arising from exposure to toxic B levels, but also to highlight the mechanisms involved in the establishment of stress signalling network(s). Even in the presence of mild or moderate levels of B excess in the growth medium, remarkably lower than those commonly adopted in previous similar experiments, the results reported here seem to indicate that B stress is promptly perceived at the root level, where it is able to cause an early and sudden burst of H_2O_2 and a resulting increase of cell membrane breakdown products. Keeping in mind the dual nature of certain ROS and of lipid peroxidation products, it remains to be understood whether their early accumulation in roots exposed to B excess could be simply regarded as priming the development of oxidative damage. This would confirm what has been previously reported in the literature, and/or serve the function of alerting antioxidant defence systems both locally and systemically.

Boron-induced activation of a ROS-scavenging enzyme, such as SOD, although episodic and exhibiting a complex behaviour in time, even preceded H_2O_2

accumulation, thus further confirming an early response activating the chain of stress signalling events, such as in response to many biotic or abiotic stress.

Boron excess in the growth medium induced a sustained increase in POD activity, apparently synchronised with the accumulation of its oxidising substrate, namely H_2O_2. Since polymerised phenolic moieties arising form POD catalysis are expected to be translocated in plant tissue, it is conceivable that they are produced in the roots mainly to fulfil a local need, which might be the lignification of specific root tissues in response to B excess.

In conclusion, none of the biochemical markers studied here can be directly connected to the differential sensitivity exhibited by Ikram and Losna towards boron excess, if not H_2O_2 accumulation in Ikram roots in response to a boron level (320 μM) to which Losna is insensitive instead. This would constitute the starting point for further studies aimed to elucidate pathways and mechanisms underlying differential responses to B excess in the two tomato genotypes.

Boron toxicity is a harsh condition to cope with in soil, then genotypic tolerance to B excess is generally considered a preferred strategy for alleviating this environmental constraint to crop production. This ultimate goal could only be achieved when B-tolerant genotypes are clearly identified and physiological mechanisms of tolerance to B toxicity are determined.

CHAPTER 4 TOMATO RESPONSE TO BORON EXCESS: THE ROLE OF GRAFTING AND ROOT MORPHOLOGY

4.1 MATERIALS AND METHODS

4.1.1 PLANT MATERIAL AND GROWTH CONDITIONS

The experiments were conducted during the 2012 late summer – fall season (Agosto– October) at Mola di Bari – Southern Italy (41°03' N, 17°4' E; 24 m asl) in a 680 m^2 polymetacrylate experimental greenhouse.

Ikram tomato genotype [*Solanum lycopersicum* (L.)] (Syngenta Seeds, Greensboro, NC, USA)], ungrafted (U), self-grafted (S) or grafted onto 'Arnold' (G) (Syngenta Seeds, Greensboro, NC, USA), an inter-specific hybrid (*S. lycopersicum* x *S. habrochaites*) rootstock, used in the present experiments, were produced by a specialized nursery. In particular, rootstock seeds were sown five days before scion seeds on 11 August 2012. Seedling were grown under protected environment in 112 cell-count polypropylene plug trays. On August 30th 2012, at two true leaf stage, rootstock and scion seedlings were grafted with the splice-tube method and held together using 2.1 or 2.3 mm polyester pipe clips and seedling support sticks (Grafting & Technology, Passatempo di Osimo, Italy). Grafted, selfgrafted and ungrafted seedlings were then transplanted at the fourth true-leaf stage, on 13 September 2012, into 4.5 L pots containing 100% perlite (Agrilit n. 3) as substrate. Pots were placed on a plastic grid, upon 5.2 m long - 1.0 m wide - 1% sloped benches, establishing a final plant density of 3.4 plants m^{-2}. Plants were trained vertically to one stem around a plastic string and, as required by common commercial practice, binding and lateral stem pruning operations were carried out on plants.

The nutrient solution was prepared with rain water containing: N (10.7 mM), P (1.6 mM), K (6.1 mM), Mg (1.9 mM), Ca (3.0 mM), S (2.9 mM), Fe (20 µM), Mn (5 µM), Zn (2 µM), B (25 µM), Cu (0.5 µM), and Mo (0.1 µM), with an electrical conductivity (EC) of 1.8 dS m^{-1}. The pH was adjusted to 5.6 using 2 M H_2SO_4. An integrated crop management approach was used to control major diseases and pests.

Minimum greenhouse temperature was set at 15 and 13 °C during day and night, respectively; ventilation temperature was 20 °C. Daily relative humidity ranged between 51-90%, with an average of 77%.

4.1.2 BORON TREATMENTS

The B treatments, 25 μM (D_0, 0,27 mg L^{-1}, considered as control), 463 μM (D_1, 5 mg L^{-1}), 925 μM (D_2, 10 mg L^{-1}) or 1,388 μM (D_3, 15 mg L^{-1}) started from seven days after transplanting (6th true leaf crop stage) and was applied by fertigation at a rate of 8.0 L $h^{-1}$to each grafting combinations until the end of the experiment. The number of fertigation events and their duration was daily scheduled to maintain a drainage percentage ranging from 40% up to 80%, to prevent the substrate ion accumulation and to avoid EC and pH changes. Collected drainage was not reused (open cycle management).

4.1.3 CHLOROPHYLL CONTENT, ROOT AND SHOOT GROWTH ANALYSIS

Before the sampling, fully developed leaf at different positions along the stem was selected for the chlorophyll content measurements which were performed by chlorophyll content meters (SPAD-502 Konica Minolta Sensing, Inc., Japan). Chlorophyll content measurements were carried out at random points (n=6) along the leaf surface area. Biometric measurements on three plants per treatment (grafting combination and B level) were performed at seven days after transplanting (T_0, right before B treatments) and then at 7 (T_1), 14 (T_2), and 21 days (T_3) after B treatment.

Shoot and root of each plant were separately harvested cutting the plant to 1 cm below the graft or the cotyledon leaves in grafted and ungrafted plants, respectively. Leaves (LDW, g) and stems (StDW, g) dry weight were determined after oven-drying at 70°C for 48 h. Shoot dry weight (SDW, g) was calculated by sum of LDW and StDW.

The roots of each plant were washed in deionized water and stored in ethanol solution until use for the root morphological analysis.

4.1.4 ROOT MORPHOLOGICAL ANALYSIS

Roots were stained with 0.1 % toluidine blue solution for 5 min and then scanned at a resolution of 300 dpi (WinRhizo STD 1600, Instruments Régent Inc., Canada). WinRhizo Pro v. 4.0 software package (Instruments Régent Inc., Canada) was used to measure root length (RL, cm) and volume (RV, cm^3). Further, root length distribution among the following root classes diameter, as defined by Bohm (1979), was obtained: very fine (VF, 0-0.5 mm), fine (F, 0.5-1mm) and large (L, >1mm). Root dry weight (RDW, g) was determined after oven-drying at 70°C for 48 h. Based on the above measurements, the root fineness (RF, root length/root

volume, cm cm^{-3}) and root tissue density (RTD, root dry weight/root volume, g cm^{-3}) were calculated.

4.1.5 STATISTICAL ANALYSIS

Treatments were arranged in a split-plot experimental design with three replicates, with the B levels (BL) in the main plots and grafting combinations (GC) in the sub-plots. All data were tested for normality (Kolmogorov–Smirnoff test) and homogeneity of variance (Levene Median test) and, where required, the data were transformed.

All parameters were analysed by three-way analysis of variance with the grafting combination and B level as main factors. Subsequently, Tukey's test was used to compare the means of all parameters of each BL and GC.

Multiple linear regression analysis between root length (dependent variable) and root dry weight, root fineness and root tissue density (independent variables) was used to evaluate the influence of the morphological components on root length.

Statistical analysis of the data was done using SPSS Statistics v. 13.0 (IBM Corp. USA) while the graphics were prepared using SigmaPlot v. 8.0 (Jandel Scientific, San Rafael, CA, USA).

4.2 RESULTS AND DISCUSSION

Shoot dry weight (SDW) was affected by both B levels and grafting combinations but these effects depended on time of exposure (significative GC x t and BL x t interactions, Table 6). In particular, SDW of plants exposed to D_0 (19.46 g) and D_1 (19.97 g) was higher than that of D_2 (15.37 g) and D_3 (14.35 g) treatments (Figure 20) 14 days forward. Furthermore, SDW variations among the different grafting combinations were also evident starting from 14 days of B exposure, showing a higher SDW in grafted plants compared to both self-grafted and ungrafted plants (Figure 20). These results suggested that the grafted tomato plants were more tolerant to B toxicity than self-grafted and, especially, ungrafted plants confirming previous data obtained in melon plants (Edelstein *et al.*, 2005).

Similar pattern was also observed for leaf and stem dry weights (Table 6; Figure 21 and 22). Indeed, B treatment affected LDW after 21 days of exposure only: D_2 and D_3 boron levels reduced by 18% and 23% their LDW, respectively compared to D_0 (significant BL x t interaction; Table 6 and Figure 21). The grafted plants pointed out higher LDW than self-grafted which, in turn, showed a higher LDW than that of the ungrafted plants, at the last two harvest times only (significant GC x t interaction; Table 6 and Figure 21).

Table 6. *Three-way ANOVA analysis (P-value) for the shoot growth and root morphological parameters of different grafting combinations (GC) of tomato plants exposed to different boron levels (BL) at diverse time of exposure (t).*

Parameters	GC	BL	t	GC x BL	GC x t	BL x t	GC x BL x t
SDW	***	***	***	NS	***	***	NS
LDW	***	***	***	NS	***	***	NS
StDW	***	***	***	NS	***	***	NS
Chl	***	***	***	NS	*	***	NS
RL	***	***	***	NS	*	***	NS
RDW	***	***	***	NS	*	***	NS
RF	***	NS	***	NS	**	NS	NS
RTD	NS	NS	***	**	***	***	***
VF	***	**	***	*	***	***	*
F	*	**	***	NS	NS	***	NS
L	NS	***	***	**	**	***	***

P values: *** p<0.001; 0.001>p>0.01; 0.01>p>0.05; NS. Not significant. SDW: shoot dry weight; LDW: leaf dry weight; StDW: stem dry weight; Chl: chlorophyll content; RL: root length; RDW: root dry weight; RF: root fineness; RTD: root tissue density; VF: length of very fine roots; F: length of fine roots; L: length of large roots.

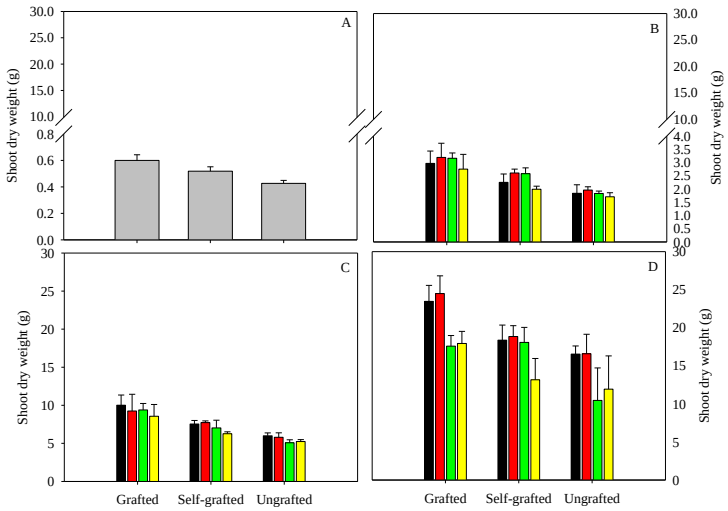


Figure 20*. Shoot dry weight of different tomato grafting combinations (Grafted, Self-grafted and Ungrafted) exposed to different increasing boron levels (■ 0 mg L^{-1}; ■ 5 mg L^{-1}, ■ 10 mg L^{-1} and ■ 15 mg L^{-1}) at diverse time of exposure (A: 0 days; B: 7 days; C: 14 days; D: 21 days).*

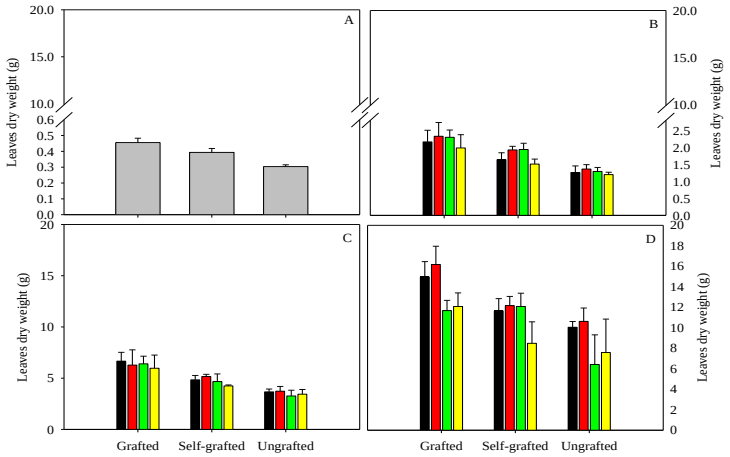


Figure 21. *Leaf dry weight of different tomato grafting combinations (Grafted, Self-grafted and Ungrafted) exposed to increasing boron levels (■ 0 mg L^{-1}; ■ 5 mg L^{-1}, ■ 10 mg L^{-1} and ■ 15 mg L^{-1}) at diverse time of exposure (A: 0 days; B: 7 days; C: 14 days; D: 21 days.)*

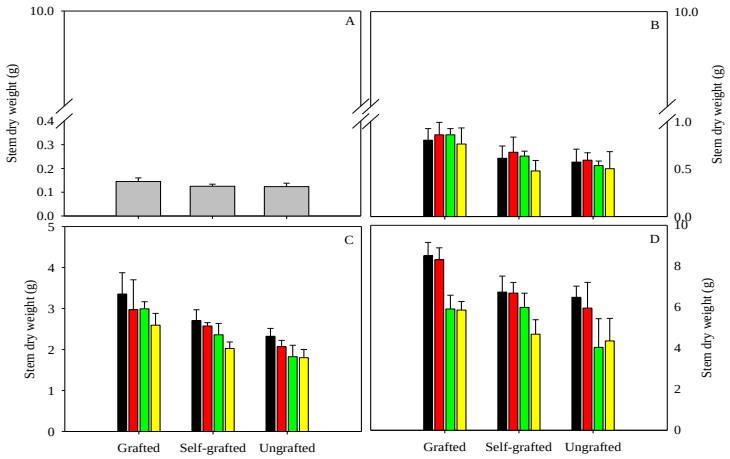


Figure 22. *Stem dry weight of different tomato grafting combinations (Grafted, Self-grafted and Ungrafted) exposed to increasing boron levels (■ 0 mg L^{-1}; ■ 5 mg L^{-1}, ■ 10 mg L^{-1} and ■ 15 mg L^{-1}) at diverse time of exposure (A: 0 days; B: 7 days; C: 14 days; D: 21 days).*

These results indicated the higher B tolerance of grafted plants which was also supported by lesser B toxicity symptoms in these plants (Figure 23), due to the delayed development and necrotic and/or chlorotic spots, especially at the margins and tips of older leaves (Nable *et al.* 1997, Bennett 1999, Reid *et al.* 2004, Paul *et al.* 1992). Stem dry weight was also modified by both grafting combinations and B levels but also with their interaction with time (Table 6 and Figure 22). In particular, the differences of StDW among the grafting combinations were already highlighted from 14 days forward of B exposure: the StDW of grafted plants (2.98 g) was significantly higher than both self-grafted (2.41 g) and ungrafted (2.00 g) plants, but these last two grafting combinations did not show any statistical difference (Figure 22). The B effect on StDW parameter was more stronger than that on LDW. Indeed, after 21 days of exposure, D_2 and D_3 treatments were able to reduce StDW by 27% and 31%, respectively, compared to control plants (Figure 22). These results were in contrast with literature data which identify the leaf as target tissue to B stress (Ardic *et al.*, 2009; Chen *et al.*, 2013; Han *et al.*, 2009; Guidi *et al.*, 2011), although Ben-Gal and Shani (2002) observed similar results in tomato.

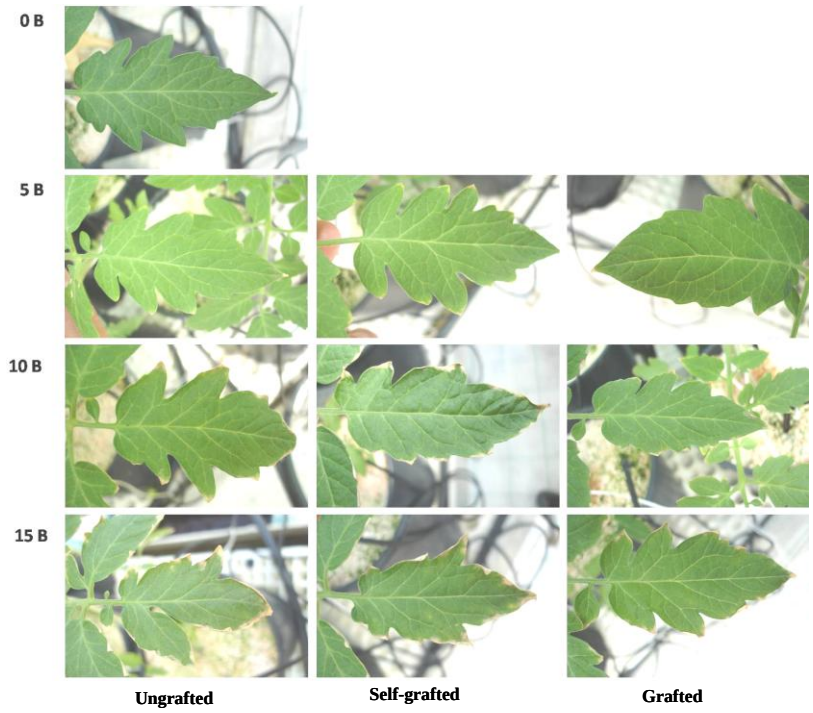


Figure 23*. Boron toxicity symptoms on leaves of different tomato grafting combinations (Grafted, Self-grafted and Ungrafted) exposed to increasing boron levels (0 mg L^{-1}; 5 mg L^{-1}, 10 mg L^{-1} and 15 mg L^{-1}) after 21 days of exposure.*

Total chlorophyll content of shoot was modified by both main factors but also with their interaction with time (Table 6). Chlorophyll content of the ungrafted plants was lower than that of other grafting combinations at all time of exposure. Furthermore, the Chl of grafted plants was higher than that of the ungrafted plants, starting from T0 of B treatment, and of the self-grafted plants, but only at the last time of exposure (4.4÷11% and 4.5%, respectively, respect to the control) (Figure 24). The B treatment reduced the chlorophyll content in tomato combinations starting from 14 days of exposure to highest B level (-7% and -11% for 14 and 21 days, respectively) while the B-induced reduction of Chl by D_2 treatment was evident at the last time of exposure only (-9%) (Figure 24). These responses were contrasting with Cervilla *et al.* (2012) which observed a boron-induced increase and no change of the chlorophyll a and b content in leaves of both B sensitive and tolerant tomato cultivars. Although, chlorophyll content reduction in leaves of other plant species was also reported (Ardic *et al.*, 2009; Chen *et al.*, 2013; Han *et al.*, 2009).

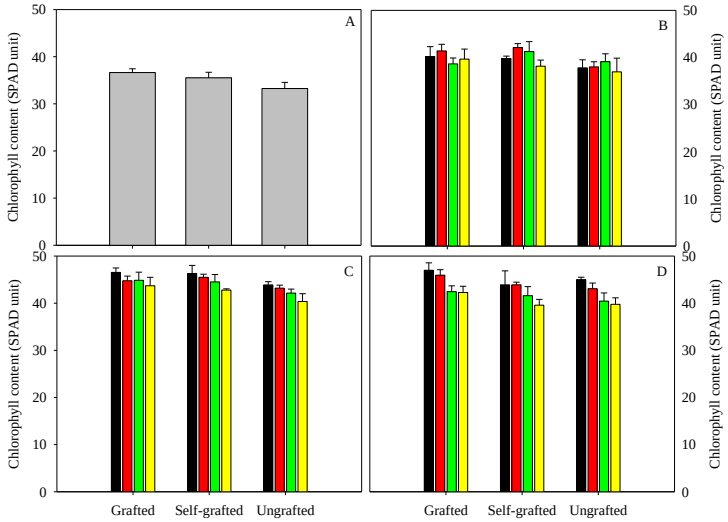


Figure 24. *Chlorophyll content of different tomato grafting combinations (Grafted, Self-grafted and Ungrafted) exposed to increasing boron levels(■ 0 mg L^{-1}; ■ 5 mg L^{-1}, ■ 10 mg L^{-1} and ■ 15 mg L^{-1}) at diverse time of exposure (A: 0 days; B: 7 days; C: 14 days; D: 21 days).*

The effect on chlorophyll content in relation to the different leaf positions of tomato grafting combinations and B levels was reported (Figures 25 and 26). Regardless B treatments, chlorophyll content varied in respect of the leaf position and grafting combinations (Figure 25). In particular, chlorophyll content changed among the five leaf positions following a well defined pattern, increasing up to the III° leaf and decreasing afterwards. This trend was maintained in all the tomato grafting combinations showing higher chlorophyll content in grafted plants respect to self-grafted and ungrafted ones (Figure 25). After 21 days of B exposure, the chlorophyll content of tomato plants was affected by both B levels and leaf position (Figure 26). In particular, in all the grafting combinations, a decreasing trend in chlorophyll content in response to the increase of B levels in the grafted and self-grafted plants, under 10 and 15 mg L^{-1} of B, respectively, and in the ungrafted plants at 5 mg L^{-1} was recorded. Concerning to the chlorophyll content among the leaf positions, interesting results were obtained. Indeed, the boron-induced chlorophyll reductions were observed in I, IV and V leaf in the ungrafted plants while at I leaf only in the grafted ones (Figure 26). Considering that the B accumulation and consequently B toxicity appeared on old leaves of melon plants (Edelstein *et al.*, 2005), these results indirectly suggested a higher B tolerance of this grafting combination compared to the ungrafted tomato plants which appeared to be more stressed under B excess exhibiting more widespread chlorophyll inhibition.

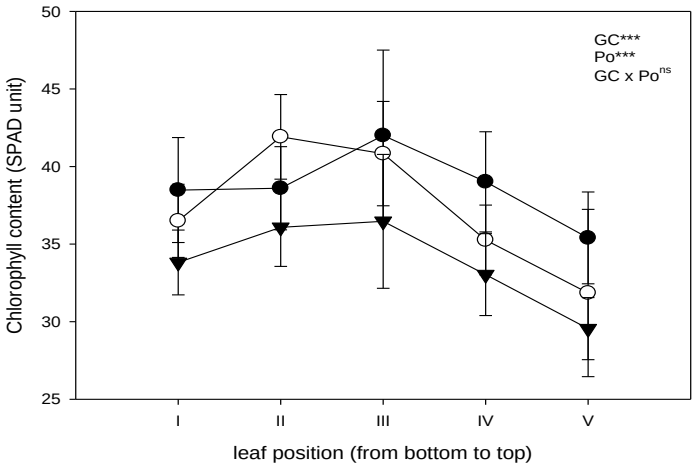


Figure 25. *Chlorophyll content in different leaf position of diverse tomato grafting combinations (●: Grafted; ○: Self-Grafted; ▼: Ungrafted). Bar errors indicated the standard deviation of n=6 means. Statistics: GC: grafting combinations; Po: leaf position; *** p<0.001; ns: not significant.*

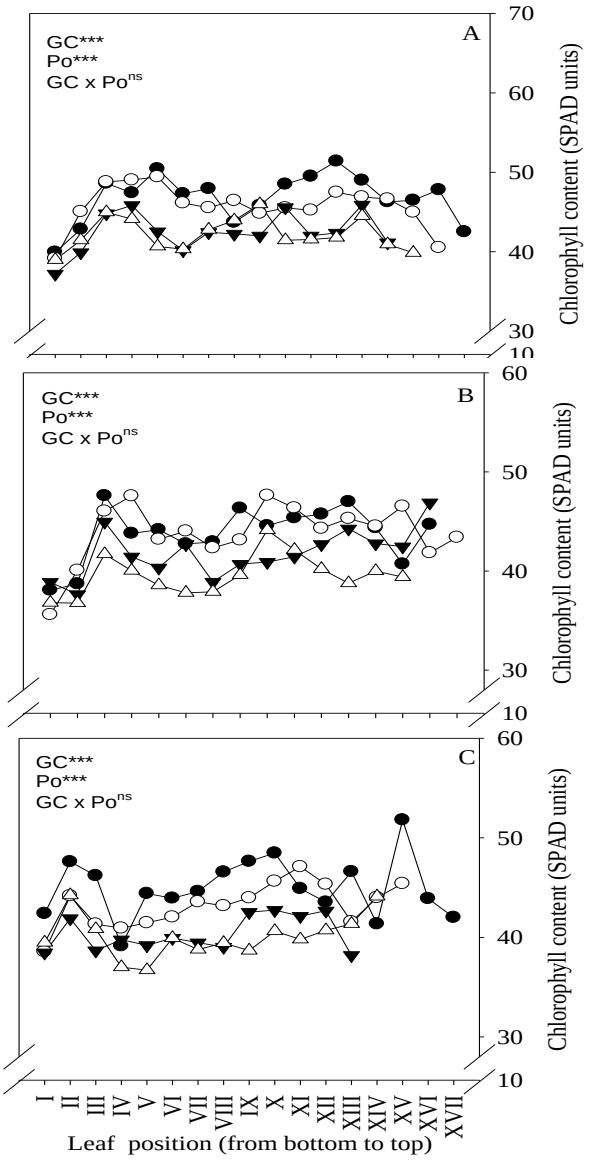


Figure 26. *Chlorophyll content in leaves at diverse position of different tomato grafting combinations (A: Grafted; B: Self-grafted; C: Ungrafted) exposed to increasing boron levels (● 0 mg L^{-1}; ○ 5 mg L^{-1}; ▼ 10 mg L^{-1}; Δ 15 mgL^{-1}) for 21 days. Bar errors indicated the standard deviation of n=3 means. Statistics: GC: grafting combinations; Po: leaf position; *** p<0.001; ns: not significant.*

The B acquisition by root system is the first step of plant control for limiting B excess and, consequently, for reducing its toxicity in cells. Root growth inhibition by B toxicity was reported in different plant species such as soybean (Kovack and Kleidus, 2008), tomato (Cervilla *et al.*, 2009), wheat (Turan *et al.*, 2009) and grapevine (Ghanati at al., 2008). Consequently, the vigorous root growth was reported as a plant strategy to B tolerance (Reid, 2010). In our study, root systems of different tomato plants in response to B treatments were evaluated by two different approaches: the "whole root analysis" and the "within root analysis". The first approach took in account diverse morphological parameters to describe the whole root system whose results are reported on Table 6 and Figures 27, 28, 29 and 30. Table 6 showed that the root length was affected by both B levels and grafting combinations although these effects were modulate by the time of exposure (significant BL x t and GC x t interactions). In particular, root length was inhibited at the last time of exposure only, while a 14%, 33% and 39% of reduction under D_1, D_2 and D_3 treatments respect to control plants was observed (Figure 27). These results confirmed the sensitivity of root growth to B toxicity as reported by Reid *et al.* (2004). Furthermore, root length of grafted plants (15830 and 26516 cm) was longer than that of self-grafted (11985 and 22511 cm) and ungrafted plants (10206 and 20047 cm) after 14 and 21 days of B exposure only (Figure 27). In particular, at the last time of exposure, root length of grafted plants exposed to D_2 and D_3 boron level was higher than that of ungrafted plants (Figure 27) confirming the B tolerance of grafted plants. Similar results induced Hayes and Reid (2004) to consider the genotypic variation in root elongation as indicator of B tolerance and Choi *et al.* (2007) to take into account root morphological changes in B tolerance strategy in barley.

However, most studies focused on the root length effects under abiotic stress did not consider the complexity of this functional trait which depends on its morphological components: root dry weight, fineness (RF) and tissue density (RTD) (Ryser, 1998) which in turn are able to modulate root length allowing plant adaptation to soil environment conditions. For example, root dry weight was the main responsible component of low nitrate-stress-induced changes in root length of citrus rootstocks (Sorgonà *et al.*, 2007); changes in RTD seemed to be responsible for plant adaptations to nutritional deficiencies (Ryser and Lambers, 1995; Hill *et al.* 2006) and flooding (Vasellati *et al.* 2001) and they were also related to the reduction in water losses at the lowest soil Ψ_w (Cruz *et al.* 1992; North and Nobel 1996; Noldt *et al.* 2001); the RF was considered to be the functional trait for the drought-tolerant herbaceous tall grass prairie species (Tucker *et al.* 2011) and bean landraces

(Abenavoli *et al.*, 2015) and it was correlated with the root's ability to take up water (Pemàn *et al.* 2006; Hernàndez *et al.* 2010).

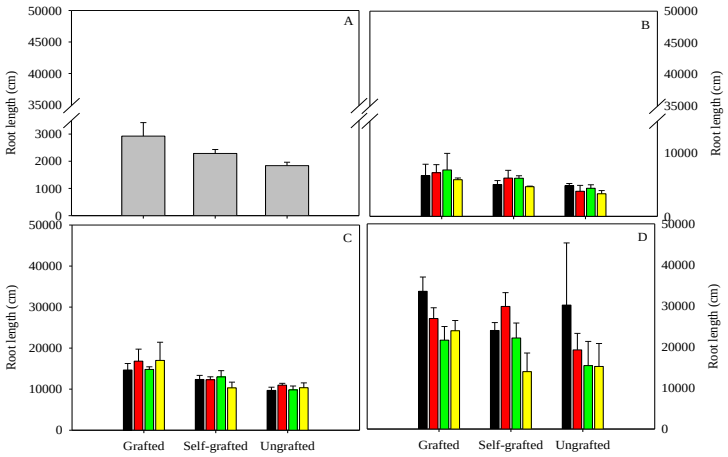


Figure 27*. Root length of different tomato grafting combinations (Grafted, Self-grafted and Ungrafted) exposed to different increasing boron levels (■ 0 mg L^{-1}; ■ 5 mg L^{-1}, ■ 10 mg L^{-1} and ■ 15 mg L^{-1}) at diverse time of exposure (A: 0 days; B: 7 days; C: 14 days; D: 21 days).*

Here, for the first time, morphological components of root length of different tomato grafting combinations in response to B toxicity were analyzed (Table 6 and Figure 28, 29 and 30). Root dry weight was highly affected by both main factors, GC and BL, and with their statistically significant interactions with the time (Table 6). In particular, the grafted and self-grafted plants showed higher RDW than ungrafted plants at the last two times of exposure (Figure 28). Further, the highest boron level reduced RDW by 23% and 27% respect to the control plants after 14 and 21 days of exposure, respectively; although, at the last time of exposure, a 27% of RDW reduction under D_2 boron level was also observed (Figure 28). Root fineness was modified by grafting combinations and time of exposure: the grafted plants pointed out a thinner root system than both self-grafted and ungrafted plants at 0, 14 and 21 days of exposure (Table 6, Figure 29). Differently from grafting combinations, B treatment did not modify the RF of tomato plants (Table 6, Figure 29). Root tissue density was not modified by both the main factors but a statistically significant GC x BL interaction was observed (Table 6). Indeed, the RTD of grafted and self-grafted plants was not affected by B treatment; while RTD of ungrafted plants was increased by D_2 B level respect to the control plants (Figure 30). However, these results were observed at 14 days of exposure only (significant GC x BL x t interaction, Table 6;

Figure 30). Overall, the results indicated that root dry weight was more affected by both grafting combinations and B treatments than root fineness and root tissue density, suggesting an important role of the RDW for the root length formation in tomato grafted/ungrafted plants exposed to B toxicity.

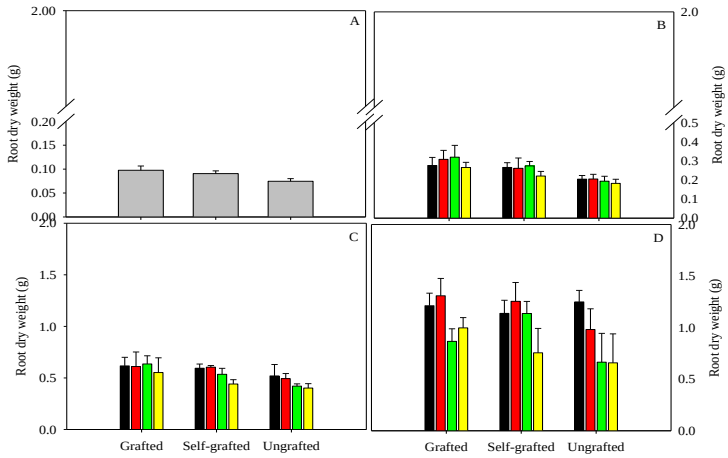


***Figure 28**. Root dry weight of different tomato grafting combinations (Grafted, Self-grafted and Ungrafted) exposed to increasing boron levels (■ 0 mg L-1; ■ 5 mg L-1, ■ 10 mg L-1 and ■ 15 mg L-1) at diverse time of exposure (A: 0 days; B: 7 days; C: 14 days; D: 21 days).*

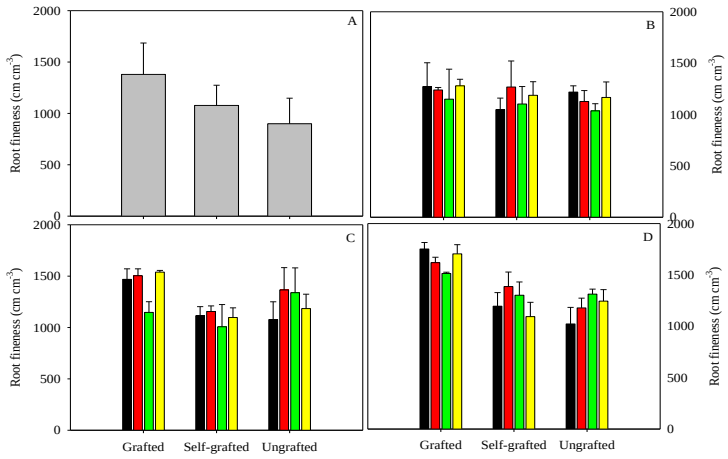


***Figure 29**. Root fineness of different tomato grafting combinations (Grafted, Self-grafted and Ungrafted) exposed to increasing boron levels (■ 0 mg L-1; ■ 5 mg L-1, ■ 10 mg L-1 and ■ 15 mg L-1) at diverse time of exposure (A: 0 days; B: 7 days; C: 14 days; D: 21days).*

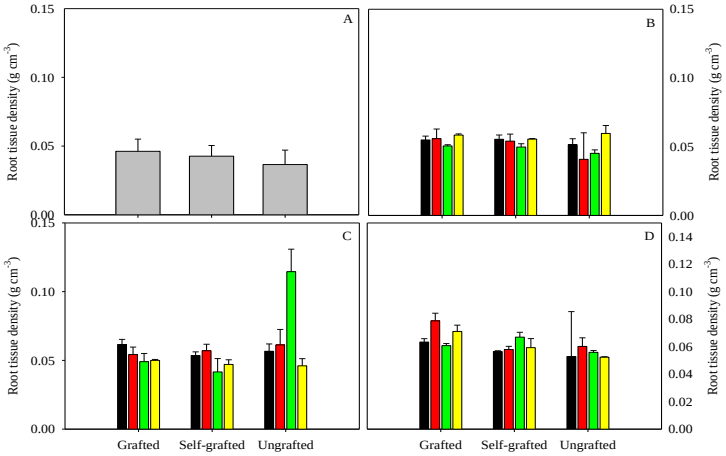


Figure 30. *Root tissue density of different tomato grafting combinations (Grafted, Self-grafted and Ungrafted) exposed to increasing boron levels (■ 0 mg L^{-1}; ■ 5 mg L^{-1}, ■ 10 mg L^{-1} and ■ 15 mg L^{-1}) at diverse time of exposure (A: 0 days; B: 7 days; C: 14 days; D: 21days).*

To confirm these results, a multiple linear regressions between root length and its morphological components was run (Figure 31). The correlation coefficient (R) of root dry weight (0.950) was higher than that of root tissue density (0.440) and root fineness (0.356) (Figure 31). Further, the results between root length and its morphological components within each B level at 21 days of exposure highlighted a marked increase of correlation coefficient of RDW with increasing of B levels, differently from the RF and RTD (Table 7 and Figure 32).

The multiple linear regressions within each grafting combinations showed that the at 21 days of exposure root length of grafted plants was determined more by root dry weight than root fineness and tissue density as well that of self-grafted plants (Table 7 and Figure 32). The root dry weight, the main responsible component in root length changes, could play a functional role in tolerance to the B toxicity. Indeed, a complex control of surcrose levels between leaf and root tip was fundamental to maintain root elongation under high B levels in barley (Choi *et al.* 2007). Conversely to the grafted plants, root length of ungrafted plants was influenced more by root tissue density and, at lesser degree, by root dry weight (Table 2 and Figure 33). Root tissue density is an adaptive trait positively correlated with the degree of lignification and cell wall thickness (Hummel *et al.* 2007) which also caused the B toxicity-induced root growth inhibition in tomato (Cervilla *et al.*, 2009) and soybean (Ghanati *et al.*, 2005).

	RDW	RF	RTD
RL of Grafted plants	0.785	0.484	0.060
RL of Self-grafted plants	0.899	0.655	0.136
RL of Ungrafted plants	0.626	0.287	0.664
RL at 0 mg L^{-1}	0.129	0.467	0.761
RL at 5 mg L^{-1}	0.894	0.442	0.072
RL at 10 mg L^{-1}	0.860	0.252	0.424
RL at 15 mg L^{-1}	0.936	0.664	0.654

Table 7. *Coefficient correlation among root length (RL) and root dry weight (RDW), root fineness (RF) and root tissue density (RTD) of different tomato grafting combinations (Grafted; Self-grafted; Ungrafted) to increasing boron levels (0 mg L^{-1}; 5 mg L^{-1}, 10 mg L^{-1} and 15 mg L^{-1}.*

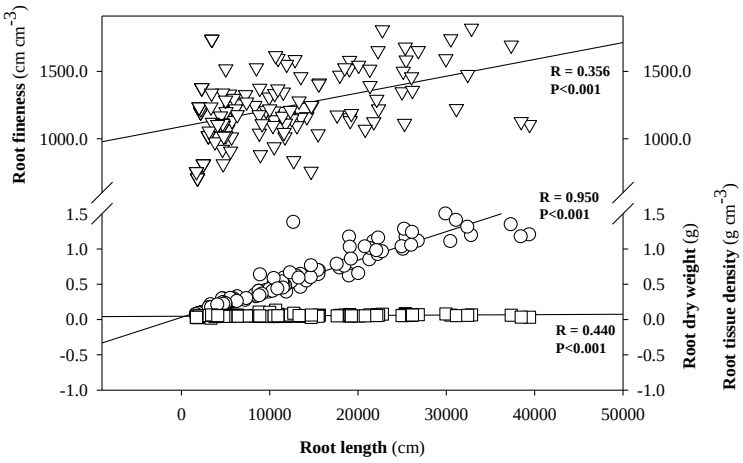


Figure 31. *Multiple regressions between root length and root dry weight (○), root fineness (▽) and root tissue density (□)of different tomato grafting combinations exposed to increasing boron levels at diverse days of exposure. R: coefficient correlation; P: probability level.*

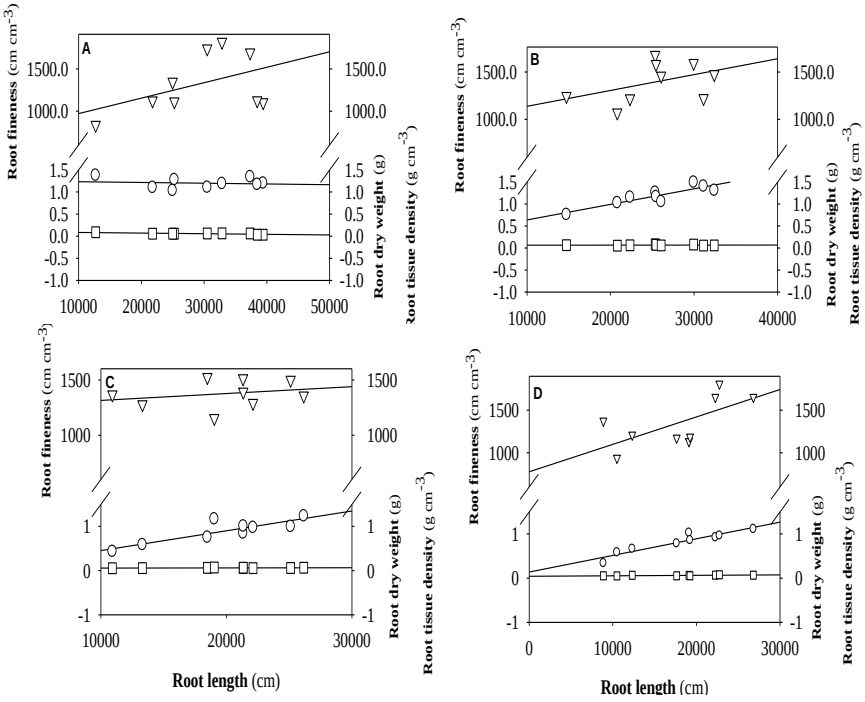


Figure 32*. Multiple regressions between root length and root dry weight (○), root fineness (▽) and root tissue density (□)of different tomato grafting combinations to increasing boron levels (A: 0 mg L^{-1}; B: 5 mg L^{-1}, C: 10 mg L^{-1} and D: 15 mg L^{-1}) at 21 days of exposure.*

The second approach took in account the variation (in term of length) of root diameter within the root system (within-root analysis) which allowed to evaluate the length of very fine (0-0.5 mm), fine (0.5-1 mm) and large roots (>1 mm) of each grafting combinations in response to B treatments. The very fine roots (0-0.5 mm) were affected by both main factors and their interactions with time of exposure (significant GC x BL, GC x t, BL x t and GC x BL x t interactions, Table 6). The comparison between the means of the different grafting combinations and B treatments at different time of exposure (GC x BL x t interaction) indicated that the different B stress-induced reduction in length of very fine roots among the grafting combinations was observed at the last time of exposure only (Figure 34).

Indeed, after 21 days of B exposure, the length of very fine roots of grafted plants was not modified by B treatments; conversely, the length of very fine roots of self-grafted (only D_3 B level) and ungrafted plants (both D_2 and D_3 B levels) was reduced by 35% and 43-52%, respectively, compared to control plants (Figure 34). The length of the fine roots (0.5-1 mm) was affected by both main factors but,

conversely to the very fine roots, there were not interactions with the time of exposure except with the boron treatment (Table 6).

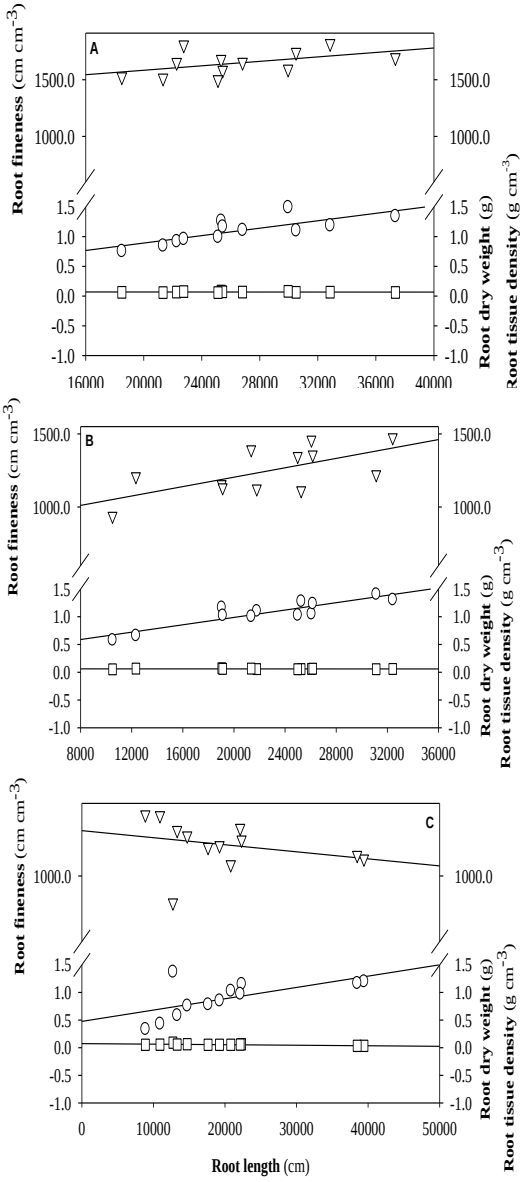


Figure 33*. Multiple regressions between root length and root dry weight (○), root fineness (▽) and root tissue density (□) of different tomato grafting combinations (A: grafted; B: self-grafted; C: ungrafted) exposed to different boron levels at 21 days of exposure.*

In particular, root system of grafted plants (5186 cm) exhibited more fine roots than both self-grafted (4918 cm) and ungrafted plants (4388 cm) (Figure 35). Further, the effects of B treatments on fine roots depended on time of exposure. Indeed, the length of the fine roots was reduced of 32% and 40% by D_2 and D_3 B levels, respectively, at the last time of exposure only (Figure 35). Finally, the length of the large roots (> 1 mm) was influenced by B treatments and its interaction with both grafting combination and time of exposure (Table 6). In particular, differently from grafted plants which did not modify their large roots in response to B treatment, the ungrafted plants pointed out a statistically significant reduction of these roots by 46%, 67% and 65% for the D_1, D_2 and D_3 treatments, respectively (Figure 36).

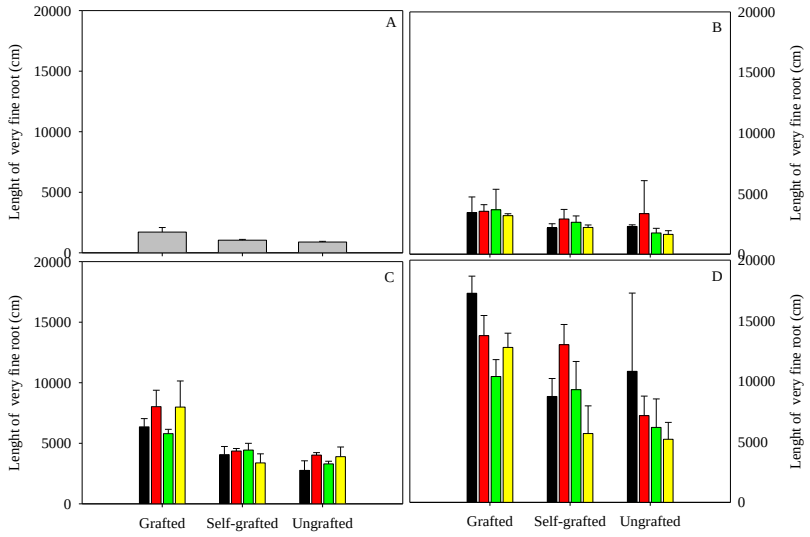


***Figure 34**. Length of very fine roots (0-0.5 mm) of different tomato grafting combinations (Grafted, Self-grafted and Ungrafted) exposed to increasing boron levels (■ 0 mg L-^{1}; ■ 5 mg L^{-1}, ■ 10 mg L-^{1} and ■ 15 mg L^{-1}) at diverse time of exposure (A: 0 days; B: 7 days; C: 14 days; D: 21days).*

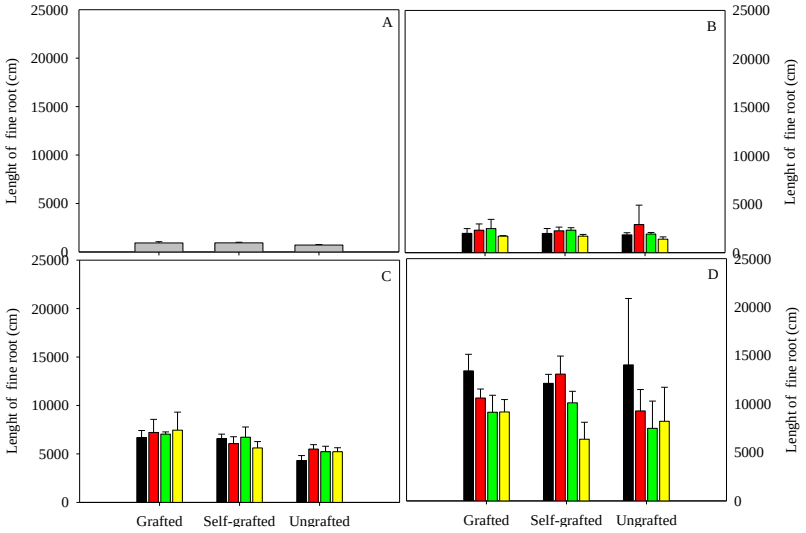


Figure 35. *Length of fine roots (0.5-1 mm) of different tomato grafting combinations (Grafted, Self-grafted and Ungrafted) exposed to increasing boron levels (■ 0 mg L-1; ■ 5 mg L^{-1}, ■ 10 mg L^{-1} and ■ 15 mg L^{-1}) at diverse time of exposure (A: 0 days; B: 7 days; C: 14 days; D: 21days).*

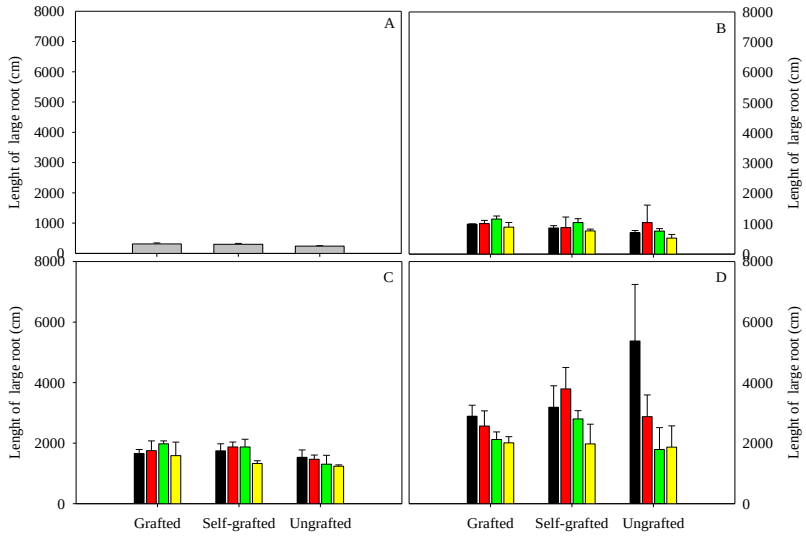


Figure 36. *Length of large roots (>-1 mm) of different tomato grafting combinations (Grafted, Self-grafted and Ungrafted) exposed to increasing boron levels (■ 0 mg L^{-1}; ■ 5 mg L^{-1}, ■ 10 mg L^{-1} and ■ 15 mg L^{-1}) at diverse time of exposure (A: 0 days; B: 7 days; C: 14 days; D: 21days).*

Overall, the results concerning the within-root analysis revealed that:

1) B treatments and, at lesser extent, grafting combinations affected the diameter classes composition of root system of tomato plants; in particular, the highest B level, especially at 21 days of exposure, reduced the length of all root diameter classes especially in large roots. This result indicated that root system became thinner and, probably, was characterized by more younger tissues in presence of high B level which was in contrast with the root sensitivity to the B excess. Indeed, Reid *et al.* (2004) reported differences in sensitivity to B of growing and mature root tissues. However, the boron efflux transporters, mainly BOR4, which pointed out an important role in the plant strategy for the B tolerance (Hayes and Reid, 2004; Sutton *et al.*, 2007; Reid, 2007) are preferentially localized around the root elongation zone (Miwa *et al.*, 2007) which is a growing tissue. Hence, it might suppose that the root system with more length of very fine roots could pointed out higher BOR4 transporters which conferred higher B tolerance.

2) The grafted plants, less sensitive grafting combination in response to B excess in terms of length of root diameter classes, maintained both higher length of the large roots, especially those very fine suggesting a more tolerance to B stress.

GENERAL CONCLUSIONS AND REMARKS

Boron (B) is an essential micronutrient in higher plants, although it is toxic in excess. Many evidences point out that several key cellular processes are sensitive to B excess, but the molecular mechanisms of B toxicity are not fully understood. Since B toxicity is more difficult to be managed in cropping systems, it is best dealt with by using B-tolerant varieties. In the last decades, remarkable insights into the potential of tolerant plants to avoid B-toxicity have been described. Although a typical visible B toxicity symptom is the appearance of chlorotic and/or necrotic spots at the margins and tips of older leaves, root system is a recurring target too and recent findings suggest its importance in B-tolerance mechanism in plant. Boron tolerance is most commonly associated with the ability to maintain a low B concentration in shoot, thus B-tolerant varieties differ from non-tolerant ones for their ability to more effectively exclude B from roots and to less translocate B to the shoot.

In this context, the present research provides a first set of physiological, biochemical and molecular responses to B-excess as well as the role of root systems in tolerance mechanism in two tomato genotypes, Ikram and Losna, contrasting in B-sensitivity.

The first study has been focused on "long and short-term boron responses in Ikram and Losna and its interaction with nitrate, important nutrient for plant growth and development. The results highlighted that after exposure to high B levels for both long (7 days) and short period (2 days), Ikram was more sensitive than Losna to B excess. Indeed, Ikram exhibited evident leaf symptom earlier than Losna accompanied by reduced chlorophyll content in shoot. Furthermore, root elongation of sensitive Ikram was immediately and more reduced as external B concentrations increased than tolerant Losna. By a deeper analysis of root morphological traits, Ikram was able to increase root tissue density (RTD), an adaptive trait positively correlated with the degree of lignification and cell wall thickness. So that, it was possible hypothesized that root growth reduction in Ikram could be caused by an increase in cell wall lignification which in turn reduced cell wall extensibility. Furthermore, since tolerance to B toxicity appeared to be correlated with lower B levels in roots and shoot, analysis of tissue B concentrations in both genotypes was also determined. Ikram showed always a higher B level compared to Losna and this pattern was evident especially in root.

Overall, the results suggested that Losna, under B excess, spend more resources in developing a more efficient root system, limiting the effects of high B on the shoot. Indeed, Losna developed a thinner and longer root system in response to B excess. This may explain both the significant increase in root length and the absence of significant variations of shoot dry weight under B excess.

Moreover, B effect on nitrate uptake was also analyzed considering that changes in root structure and distribution are usually accompanied by changes in mineral nutrition. A significant inhibitory effect on net nitrate uptake rate (NNUR) was observed only in Ikram already at lower B toxic concentration, accompanied by a drop of *NRT2./NAR2.1* expression, genes strictly involved in the regulation of NO_3^- uptake. Nevertheless, this trend was evident in Losna only at full induction time, where a higher nitrate uptake mirrored high *NRT2./NAR2.1* transcript abundance also under toxic B level. At first glance, this result could be explained by an increase of pmH^+-ATPase, at biochemical and molecular level, which could lead to an increase of nitrate uptake across the plasma membrane.

To verify the molecular mechanism of different sensitivity to B toxicity between tomato genotypes, the expression of two main classes of B transporters such as NIP5;1, responsible of bidirectional movement of boric acid; BOR1 a facilitated efflux of boric acid out of the cell into the cell wall; and BOR4, a B-permeable channels responsible for directional B export from the roots to the soil, involved in B tolerance, was analyzed. As expected, *NIP5;1* and *BOR1* expression patterns at B toxic concentrations did not show differences between tomato genotypes and appeared rather down-regulated by B excess. In contrast, *BOR4* expression was higher in Losna under B excess, compared to Ikram.

So that, the most important mechanism able to explain the tolerance of Losna is based on B efflux from roots. Recently, active borate efflux through BOR-type transporters strongly dependent on borate permeability and needed energy input to actively extrude H^+ (*via* H^+-ATPase) to maintain the electrical driving force for borate efflux was postulated. Electrophysiological experiments, performed in presence of stimulators or inhibitors of H^+-ATPase in roots of both tomato genotypes, suggested the involvement of this enzyme to sustain borate efflux. Indeed, B-tolerant Losna showed a higher membrane potential hyperpolarization than Ikram in response to B. Thus, the first hypothesis of B-tolerance in tomato was that borate efflux was driven by electrogenic proton efflux *via* H^+-ATPase. Surprisingly, in presence of vanadate, a metabolic inhibitor of electrogenic H^+ pump, Losna showed a low membrane hyperpolarization yet. This suggested that, electrogenic proton efflux other than H^+ ATPase was necessary to maintain the gradients for borate efflux in Losna genotypes. In this respect additional experiments have to be performed to demonstrate this hypothesis. Further, the B toxicity tolerance mechanisms described here are naturally occurring in root system, and this provides an opportunity to explore the basis for their evolution.

Since Boron (B) toxicity induces oxidative stress and alterations in the photosynthetic process, the second study was focused on short term responses of

several antioxidants in the root system of tomato genotypes to highlight the mechanisms involved in the establishment of B stress signalling network(s). The results suggested that B-induced an episodic and complex activation of SOD, a ROS-scavenging enzyme, even preceded by H_2O_2 accumulation, thus further confirming an early response activating the chain of stress signalling events, such as reported in many biotic or abiotic stresses. Boron excess in the growth medium induced a sustained increase in POD activity, apparently synchronised with the accumulation of its oxidising substrate, namely H_2O_2. Since polymerised phenolic moieties arising from POD catalysis are expected to be translocated in plant tissue, it is conceivable that they are produced in the roots mainly to fulfil a local need, which might be the lignification of specific root tissues in response to B excess. In conclusion, none of the biochemical markers analyzed can be directly connected to the differential sensitivity exhibited by Ikram and Losna towards B excess, if not H_2O_2 accumulation in Ikram roots in response to a B level (320 μM) to which Losna is insensitive instead. This would constitute the starting point for further studies aimed to elucidate pathways and mechanisms underlying differential responses to B excess in the two tomato genotypes.

Boron toxicity represents one of the most feared abiotic stresses limiting vegetable production in several areas of the Mediterranean Basin, and often it is associated to salinity stress. In the third part of the thesis, a study to evaluate the role of tomato grafting in enhancing the tomato plant tolerance to boron excess was conducted. Grafting technique is increasing in Italy and many others country being an effective practice especially to control some of the major soil-borne pathogens and to alleviate the effect of soil salinization. In this respect, tomato (*Solanum lycopersicum* L. cv. Ikram) plants ungrafted or grafted onto inter-specific tomato hybrid rootstocks (*S. lycopersicum* x *S. habrochaites* - 'Arnold' and 'Big Power') or self-grafted were studied to evaluate their effects on the seedling growth and root system responses to B excess.

The results suggested that the grafted tomato plants were more tolerant to B toxicity than self-grafted and, especially, ungrafted plants. The higher B tolerance of grafted plants was confirmed by both lesser reduction of shoot dry weight and higher chlorophyll content and consequently a delayed development and necrotic and/or chlorotic spots, especially at the margins and tips of older leaves. Further, this effect was also evident among the leaf position, where the ungrafted plants appeared to be more stressed under B excess than grafted, exhibiting more widespread chlorophyll inhibition. Besides, the morphological components of root length in response to B toxicity were analyzed applying two different approaches: "whole root analysis" and "within root analysis".

The first results indicated that root dry weight was more affected by both grafting combinations and B treatments than root fineness and root tissue density, suggesting its important role for root elongation in tomato grafted plants exposed to B toxicity. Conversely, root length of ungrafted plants was more influenced by root tissue density and, at lesser degree, by root dry weight. Root tissue density is an adaptive trait positively correlated with the degree of lignification and cell wall thickness which also caused the B toxicity-induced root growth inhibition.

The results concerning the "within-root analysis" revealed that B treatments and, at lesser extent, the grafting affected the diameter classes of root system. In particular, the highest B level reduced the length of all root diameter classes, indicating a thinning root system, characterized by younger tissues in presence of high B level. These observations, in contrast with the root sensitivity to B excess, denoted that the grafted plants maintained a higher elongation of both large and very fine roots, suggesting a more tolerance to B excess stress.

In conclusion, the root system could really play a pivotal role in tomato B toxicity tolerance mechanism. Further, it is also possible to suggest that the use of grafting on particular rootstocks can represent an effective strategy to cope high B concentration in soil.

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