DISTRIBUTION OF CHROMIUM AND ITS EFFECT ON MORPHOLOGY AND ANATOMY OF *BRASSICA JUNCEA* (INDIAN MUSTARD) AND SOIL MICROORGANISMS

by

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ABSTRACT

The present study was conducted to evaluate the growth response of *Brassica juncea* (Indian mustard) and soil microorganisms to two forms of chromium (Cr) and to study mechanism(s) involved in Cr binding and sequestration by the plant. The chemical speciation of Cr in rhizosphere soil was also investigated in this thesis.

B. juncea was grown under greenhouse conditions in field-moist or air-dried soils, amended with either Cr (III) or Cr (VI). The plant concentrated approximately 200 mg Cr per kg of root dry weight (DW) and 6 mg Cr per kg of shoot DW in the Cr (III)-amended soil. In the Cr (VI)-amended soil, the plant accumulated approximately 390 mg Cr per kg of root DW and 20 mg Cr per kg of shoot DW. In general, the plant was tolerant of both Cr treatments. Cr (VI) appeared to be more toxic to both soil microorganisms and plant morphology and anatomy than Cr (III) and root growth and anatomical characteristics were inhibited to a greater extent than those of shoots. Soil chemical analyses and X-ray absorption near-edge spectroscopy (XANES) detected Cr (III) species in Cr (VI) treatment. The XANES and X-ray microprobe spectroscopy data for the plant tissues revealed nearly complete conversion of Cr (VI) to Cr (III) in the roots, where it was accumulated preferentially in epidermal and cortical cells as Cr (III)-acetate (72%), while, in the leaves, it was concentrated in epidermal and spongy mesophyll cells as Cr (III)-oxalate (81%). The ability of *B. juncea* to tolerate and detoxify Cr (VI) within the roots makes this plant a potential candidate for phytostabilization.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	percent
°	degrees
°C	degrees Celcius
µg	microgram
µL	microliter
µm	micrometer
µM	micromolar
Al_p and Fe_p	extractable with sodium pyrophosphate
CEC	cation exchange capacity
cm	centimeter
cmolc	centimoles of charge
Eh	redox potential
g	gram
h	hour
ha	hectare
ID	internal diameter
keV	kilo electron volts
kg	kilogram
kV	kilovolt
L	liter
M	molar
Me	metal
meq	milliequivalent
mg	milligram
min	minutes
mL	milliliter
mm	millimeter

mM	millimolar
mm^2	millimeter square
n	sample size
nm	nanometer
p	probability
psi	pounds per square inch
rpm	revolutions per minute
sec	second
spp.	species
α	significance level

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1.0. INTRODUCTION

Soils naturally contain heavy metals as a result of proximity to mineral outcrops or ore bodies and/or anthropogenically as a result of industrial activities (Baker *et al.*, 1994a). Chromium is a major soil contaminant due to its use in metallurgy, tanneries, leathers, dyes, textile, and wood preservation (Adriano, 1986). In soils, Cr is present in two stable oxidation states: Cr (III) and Cr (VI). Cr (III) exists in soil mostly as insoluble oxides and hydroxides. It is also associated with soil minerals and organic matter. Therefore, it is considered stable in soils. However, the potential for oxidation of Cr (III) to Cr (VI) can make the former almost as hazardous as the latter.

Contrary to Cr (III), Cr (VI) is more soluble and hence mobile in soils. As a result, Cr (VI) can enter the food chain through either leaching into groundwater or absorption by plants. In addition, both Cr (III) and Cr (VI) produce carcinogenic activity in animals, including humans (Norseth, 1981). In particular, both Cr forms can inhibit synthesis of deoxyribonucleic acid (DNA) (Tamino *et al.*, 1981). Cr (VI) crosses cell membranes more easily than Cr (III) (Arslan *et al.*, 1987). However, once in the system, the reduction of Cr (VI) to Cr (III) or Cr (V) takes place (Norseth, 1981; Micera and Dessi, 1988; Liu *et al.*, 1995). Formed Cr (III) and Cr (V) complexes react with hydrogen peroxide (H_2O_2), generating the highly toxic free hydroxyl ($^{\cdot}OH$) radicals, which trigger DNA oxidative damage

(Shi and Dalal, 1990). For all of the above-mentioned reasons, there is concern relating to soil pollution issues which justifies the importance of cleaning up Cr (III, VI)-contaminated soils.

Several methods have been used to treat metal-contaminated soils including landfilling (excavation, transport and deposition of contaminated soil in a permitted hazardous waste landfill), solidification/stabilization or fixation (chemical processing of soil to immobilize metals), and soil flushing and washing or leaching (use of acid solutions or other leachants to desorb and leach metals from soil) (Salt *et al.*, 1995). However, these technologies are extremely expensive and very disruptive to the site and the environment (Lasat, 2002). Phytoremediation (use of plants in remediation) has been employed for years as an alternative to the above approaches. It has become one of the most popular techniques used in remediation because plants can be aesthetically pleasing, economically viable, and non-destructive to the environment. Another advantage of phytoremediation is the potential recovery of the valuable metal components from the contaminated biomass through repeated harvests (Chaney *et al.*, 1997). However, one of the drawbacks of phytoremediation is the potential contamination of the food chain via wildlife.

The phytoremediation concept is based on the ability of particular plants to extract, concentrate, and/or degrade contaminants (Baker and Brooks, 1989).

According to Reeves *et al.* (1995), hyperaccumulators are those plants which leaves can accumulate more than 100 mg cadmium (Cd) or selenium (Se) kg^{-1} (DW), more than 1,000 mg lead (Pb), nickel (Ni), cobalt (Co), Cr, or copper (Cu) kg^{-1} (DW), and more than 10,000 mg iron (Fe), manganese (Mn), or zinc (Zn) kg^{-1} (DW) when grown in metal-rich soils. Heavy metal resistant plants can be planted and left to grow for several seasons before they are harvested, incinerated and the ash safely disposed of (Sellers, 1998).

To be effective remediators of soil heavy metals, plants must also have the ability to metabolize metals and maintain growth and development. Hyperaccumulators, unlike sensitive plants, have developed unique (metal and plant-specific) resistance mechanisms to defend their cellular activity and structures against metal stresses. These mechanisms include metal chelation with appropriate high-affinity ligands (preferentially low-molecular-weight organic acids originating from root exudates), biotransformation with reductants and subsequent compartmentalization either in the cytoplasm or in a sub-cellular organelle, i.e., the vacuole (Salt *et al.*, 1998). Nevertheless, further knowledge on how these mechanisms and the mechanisms of metal uptake and translocation function is required to enhance the effectiveness of phytoremediation.

Brassica juncea (Indian mustard) is a member of Kingdom Plantae (plants), Subkingdom Tracheobionta (vascular plants), Superdivision Spermatophyta (seed plants), Division Magnoliophyta (flowering plants), Class Magnoliopsida (dicotyledons), Subclass Dilleniidae, Order Capparales, and Family Brassicaceae (mustard). It is referred to as brown mustard or Indian mustard (USDA NRCS, 2003). *B. juncea* has been widely employed in phytoremediation because of its remarkable capacity to accumulate high levels of heavy metals, including Ni, Zn, Cu, and Pb. For example, its shoots have been reported to take up 308,600 mg Ni kg^{-1} plant (DW) and 172,300 mg Zn kg^{-1} plant (DW) in a fertilized sand/Perlite mixture supplemented with 100 mg Me kg^{-1} soil (Kumar *et al.,* 1995). *B. juncea* is also considered a Cr hyperaccumulator. For example, in a study by Salt *et al.* (1995), when grown in a hydroponic culture system and relatively low Cr concentration, i.e., 0.4 mg L^{-1}, plant seedlings accumulated 2,194 mg kg^{-1} DW in their roots in a single cropping. Once taken up by the plant, Cr is generally retained in roots rather than in shoots. In a study by Shahandeh and Hossner (2000), *B. juncea*, supplied with 100 mg Cr (III) or Cr (VI) kg^{-1} soil, accumulated 191 and 4.6 mg Cr (III) kg^{-1} and 431 and 44.7 mg Cr (VI) kg^{-1} in plant roots and shoots, respectively. Other benefits of using this species in phytoremediation is its ability to grow quickly (matures 8-10 weeks after sowing) and to produce a high amount of biomass in relatively short periods of time (18 t ha^{-1} in 2.5 months) (Blaylock *et al.*, 1997).

The effectiveness of *B. juncea* in removal of Cr from polluted soils greatly depends both on Cr bioavailability and the ability of the plant to tolerate the metal. However, information concerning the Cr toxicity and metal sequestration in this species is limited. The present study was undertaken to gain a better general understanding of the mechanism(s) involved in Cr binding and tolerance by plants. A further goal of this research was to examine the effects of Cr on soil microbial activity as well as to investigate growth and developmental responses of *B. juncea* when grown in different Cr and soil treatments. The rhizosphere zone of the plant (soil-root interface), which serves as the main entry point of metals into roots, was of special interest. The particular objectives of this study were: (i) to determine the effect of Cr (III, VI) and soil (field-moist, air-dried) treatments on solubility and, hence, availability of Cr to the plant; (ii) to assess speciation of Cr in polluted rhizosphere and bulk soils; (iii) to establish whether Cr affects activity of soil microorganisms; (iv) to determine the extent to which *B. juncea* can absorb Cr and to identify the form of accumulated and translocated metal, its chemical speciation and distribution within the plant; (v) to observe any Cr toxicity in the plant at macroscopic (visible stress and root and shoot growth) and microscopic (stem, root, and leaf cells) levels; and (vi) to evaluate how low-molecular-weight organic acids in root exudates of *B. juncea* are influenced by different developmental stages of the plant and Cr application. It is hoped that knowledge gained from this study will help to elucidate the role of plants in soil

remediation and, additionally, to potentially provide knowledge of particular heavy metal species that are detrimental to plant growth and development.

2.0. BACKGROUND

2.1. Distribution of Chromium in Soil

2.1.1. Sources of soil chromium

Chromium naturally occurs in minerals and rocks and can be released into the soil environment by their weathering and erosion (Adriano, 1986). Minerals such as chromite ($FeCr_2O_4$) and crokoite ($PbCrO_4$) contain high amounts of Cr; however, they are highly insoluble and, thus, sparse in the soil environment. Soils naturally high in Cr can be found where parent material is derived from serpentine rocks. In particular, the concentration of Cr ranges from 5 to 1,000 mg kg^{-1} in natural soils, while it can reach 125,000 mg kg^{-1} in serpentine soils (Adriano, 1986).

Anthropogenic inputs of Cr into soils can be a result of both industrial activities, such as production of alloys, leather tanning, electroplating, and dyeing, and agricultural sources, including application of pesticides, fertilizers, and manures (Nriagu and Kabir, 1995). Moreover, improper handling and storage of Cr-containing wood preserves can result in the transfer of large metal quantities to local soils (Bamwoya *et al.*, 1991). Weathering of treated lumber exposed to acidic rainfall can also release Cr (Warner and Solomon, 1990). It is estimated that wastes containing more than 5,000 ton of Cr in various forms are dumped annually onto Canadian soils (The National Contaminated Sites

Remediation Program, 1996). Since Cr is not biodegradable, it has infinite persistence in the soil environment (Bartlett, 1991).

2.1.2. Chromium speciation, mobility and availability

Chromium is a unique heavy metal, because it exists in soil environments in two stable valence states, i.e., Cr (III) and Cr (VI), with contrasting solubility, mobility, and, consequently, toxicity (Bartlett and James, 1988). The behavior of these two forms of Cr in the soil environment strongly correlates with their potential for precipitation/dissolution, sorption/desorption, and oxidation/reduction.

The main species of Cr (III) in soils are Cr^{3+} at pH < 3.6, $[Cr(H_2O)_6]^{3+}$ and $[Cr(H_2O)_5OH]^{2+}$ at pH 4, $Cr(OH)_3$ at pH from 6.3 to 11.5, and $[Cr(OH)_4]^-$ at pH above 12 (Rai *et al.*, 1989). Cr (III), like other first-row transition metals, has a strong tendency to form octahedral coordination compounds, including complexes and chelates with oxygen (O), sulfur (S), and nitrogen (N)-containing ligands (Nakayama *et al.,* 1981). As a result, Cr (III) solubility in soils is restricted due to formation of Cr oxides, hydroxides, and phosphates and mixed complexes with Fe. Moreover, Cr (III) greatly binds to clay particles and soil organic matter and, as the pH increases, Cr (III) adsorption increases (Griffin *et al.,* 1977) only for clay-sized minerals containing pH-dependent surface charges such as the oxides of Fe, Al and Si, but not for phyllosilicates (Brady and Weil,

2002). Therefore, Cr (III) is considered immobile, unavailable and, thus, relatively harmless in soils.

Under certain soil conditions, however, Cr (III) may be oxidized to Cr (VI), which may leach from the soil system, thereby leading to serious environmental consequences (Bartlett and James, 1979). According to Cary (1982), oxygen does not react appreciably with Cr (III). The most likely oxidant of Cr (III) in soil is Mn (IV) oxide acting as an electron acceptor and a link between Cr (III) and the oxygen in the atmosphere (Cary, 1982; Bartlett and James, 1988). Although soil microorganisms do not appear to be required for the oxidation (Ross and Bartlett, 1981), they might be involved indirectly, because all microbially-mediated transformations in soil seem to result in pH and Eh shifts (Bartlett, 1986a). The oxidation of Cr (III) by soil manganese oxides is controlled by the surface characteristics of the oxides and the availability of the Cr (III) to the surface (Bartlett and James, 1988). Manganese oxides typically accumulate on the surfaces of clays and Fe oxides (Bartlett and James, 1979). Manganese minerals (oxides, silicates, and carbonates) have large surface areas and tend to have negative charges at all but acidic conditions. These properties are associated with their high absorptive capacities, particularly for heavy metals (Bartlett and James, 1979).

Some adsorbed Cr (III) species, tightly bound to humic substances or recently precipitated Cr hydroxides, can be readily extracted by salts of low-molecular-weight organic acids that appear to be the most likely vehicles by which Cr (III) moves toward oxidizing Mn surfaces (Bartlett, 1986b). The oxidation rate of organic Cr (III) compounds is slower than that of freshly precipitated Cr (III) (James and Bartlett, 1983). The reason for this is the presence of extraneous organic materials, containing carboxyl groups and phosphates, which can form strong complexes with Mn (III) and cause reverse dismutation to take place via the following reaction:

$$Mn^{2+} + MnO_2 + 4H^+ \leftrightarrow 2Mn^{3+} + 2H_2O$$

Mn (III) can further oxidize the organic carbon (C) that it is holding and become reduced to Mn (II), thereby making Cr (III) oxidation impossible (Bartlett, 1986a).

The chemistry of Cr (VI) is very different from that of Cr (III). Cr (VI) forms a number of anions such as $HCrO_4^-$ (hydrochromate), $Cr_2O_7^{2-}$ (dichromate) and CrO_4^{2-} (chromate), the last being the predominant form at pH > 6 (Rai *et al.,* 1989). Cr (VI) compounds are very unstable and soluble in both acid and alkaline soils (Adriano, 1986). They do not readily adsorb to surfaces. If they do, mineral solids that contain inorganic hydroxyl groups, including Fe and aluminum (Al) oxides, kaolinite, and montmorillonite, are among the main adsorbents of Cr (VI) (Rai *et al.,* 1989). Adsorption increases with decreasing pH as a result of protonation of the surface hydroxyl sites (Rai *et al.,* 1989). The

intensity of adsorption to soil oxides and clay particles also depends on the presence of competing ligands, such as phosphate, carbonate and sulfate (Adriano, 1986). Therefore, liming and application of phosphate fertilizers, for example, are practiced to remobilize adsorbed Cr (VI) (Bartlett and James, 1988).

The Cr (VI) ion is a stronger oxidizing agent than Cr (III) because of its large positive reduction potential and tetrahedral coordination (Nriagu and Kabir, 1995). The high oxidizing potential and solubility make Cr (VI) more mobile and, hence, toxic than Cr (III). On the other hand, the high oxidizing potential of Cr (VI) means it is easily reduced to Cr (III), when electron donors, such as soil humic compounds (fulvic and humic acid), non-humic substances (low-molecular-weight organic acids, carbohydrates and proteins), aqueous inorganic species (Fe (III) and sulfides) and common soil minerals (kaolinite and montmorillonite) are present in soil (Bartlett and Kimble, 1976; Cary, 1982). Another factor that affects the rate and extent of Cr (VI) reduction is pH. Generally, because hydrogen is consumed in the reduction reaction, it mainly occurs in acid soils and under both aerobic and anaerobic conditions. In addition, soil microbial activity may influence Cr (VI) mobilization both directly via release of special enzymes-reductases (Salt *et al.*, 1998) or indirectly via production of chelates, including low-molecular-weight organic acids (Bartlett,

1991) or depletion of soil oxygen levels which tends to lower soil pH (Losi *et al.*, 1994).

2.2. Chromium and Soil Microorganisms

2.2.1. Chromium inhibitory effects

Probably because of its low solubility and high tendency to undergo precipitation and complexation reactions, Cr (III) is not readily taken up by microbial cells compared with the more mobile Cr (VI) that can easily penetrate cell membranes (Arslan *et al.*, 1987). Therefore, the former is not considered to be particularly toxic to soil microorganisms, while the latter is. Once inside the cytoplasm, Cr (VI) is reduced, but its toxicity most likely results from oxidation of cell components (Arslan *et al.*, 1987). However, the ability of Cr (III) to become oxidized and produce Cr (VI), although for a short time, might be responsible for inhibitory effects of this form of Cr on soil microbial activity (Bartlett and James, 1988). For example, Ross *et al.* (1981) reported that addition of 10 and 100 mg Cr (VI) kg^{-1} soil and 100 mg Cr (III) kg^{-1} soil significantly reduced carbon dioxide (CO_2) evolution when added to loam and fine sandy loam soils. Moreover, in spite of the fact that extractable Cr (VI) disappeared for the 3-week duration of the study, the CO_2 evolution did not increase (Ross *et al.*, 1981). The results of other short- as well as long-term experiments indicate that other effects including species abundance, nitrogen biotransformation, and enzyme (phosphatase,

sulphatase, arylsulphatase, and urease) activities are also inhibited in soil microbes, when the added concentrations are 25 to 100 mg Cr (III) kg^{-1} soil and 1 to 10 mg Cr (VI) kg^{-1} soil (Tabatabai, 1977; Bollag and Barabasz, 1979; Doelman and Haanstra, 1984, 1986; Yadav *et al.*, 1986; Speir *et al.*, 1995).

2.2.2. Microbial reduction of Cr (VI)

Bacteria that are able to reduce Cr (VI) to Cr (III) have been reported by several researchers (Horitsu *et al.*, 1987; Bopp and Ehrlich, 1988; Wang *et al.*, 1989). Representatives of the genera *Aeromonas*, *Escherichia*, *Pseudomonas*, and *Enterobacter* are among microorganisms capable of Cr (VI) reduction (Kvasnikov *et al.*, 1988). The reduction is carried out either by cell membranes or by soluble proteins (Chen and Hao, 1998). For example, membrane-bound Cr (VI) reductases have been found in *Pseudomonas fluorescens* (Bopp and Ehrlich, 1988) and *Enterobacter cloacae* (Wang *et al.*, 1989). Such enzymatic mechanisms ensure that this process takes place at the surface of bacterial cells, where Cr (III) hydroxides precipitate, thereby protecting cells from the toxicity of Cr (VI) (Wang *et al.*, 1989). In addition, depending on growth conditions and type of Cr (VI)-reducing bacteria, some organic substrates including amino acids (peptone, glucose and lactose), salts of organic acids (propionate, acetate, malate, succinate, citrate, pyruvate, and lactate), ethanol, and glycerol, might serve as electron donors for the Cr (VI) reduction (Chen and Hao, 1998). However, reduction of Cr (VI) is generally slower than its uptake in

bacterial systems (Ohtake and Silver, 1994); as a result, in order for detoxification to occur, bacteria must be able to tolerate the toxicity of Cr (VI). It has been demonstrated that Cr (VI) resistance is plasmid-associated. The genes for a hydrophobic polypeptide, i.e., chromogranin A (ChrA), have been identified in *Pseudomonas aeruginosa* and *Alcaligenes eutrophus* and thought to be responsible for the outward translocation of Cr (VI) anions (Cervantes and Silver, 1992). There is also evidence that sulfate, which is chemically-analogous to the Cr (VI) anion, can competitively inhibit Cr (VI) uptake in microbial systems, leading to lower accumulation of Cr (VI) and bacterial resistance (Ohtake *et al.*, 1987). Yet another mechanism of the Cr (VI) resistance has been discovered in *Pseudomonas ambigua* (Horitsu *et al.*, 1983). The particular surface structure (a thick envelope) of the bacterial membrane results in its low permeability to Cr (VI). The unique abilities of bacteria to reduce and withstand toxic Cr (VI) may be useful for bioremediation, an emerging biological method to remediate Cr-contaminated soils.

2.3. Heavy Metals and Plants

2.3.1. Metal toxicity

Plants require both macronutrients and micronutrients for their growth. Some trace elements (heavy metals) including Cu, Mn, and Fe are important in plant physiological processes (Baker and Brooks, 1989), where they can act as

activators of a number of enzymes in photosynthesis, respiration, oxidative phosphorylation, and DNA, ribonucleic acid (RNA) and protein syntheses (Salisbury and Ross, 1992). However, other metals such as Co, Ni, and Al, are not considered to have any essential function in plants (Baker and Brooks, 1989).

Whether essential or non-essential for plant nutrition, heavy metals may become toxic to plants at relatively low concentrations. For example, Rhoads (1971) observed toxicity in *Nicotiana tabacum* (tobacco) irrigated with water containing greater than 1.5 mg Fe kg^{-1} soil. Excessive heavy metal ions may induce a series of biochemical and physiological effects such as membrane damage, alteration of enzyme activity and hormone balance, deficiency of essential nutrients and water, and inhibition of photosynthesis and root growth (Barcelo and Porshenrieder, 1990). The most commonly observed symptoms of phytotoxicity are chlorosis and stunting. The chlorosis from excess of heavy metals such as Zn, Cu, Ni, and Cd has often been attributed to failure in Fe metabolism of treated plants (Foy *et al.,* 1978). Iron deficiency in plants results in inhibition of both chloroplast development and chlorophyll biosynthesis (Imsande, 1998).

The stunting effects of metals can be commonly due to either a specific toxicity of a metal to a plant or inhibition of root penetration into the soil. In higher

land plants, the root is the first organ to contact the metal, so that toxicity is first experienced there. Inhibition of root elongation leads to decrease in uptake of nutrients and, consequently, in plant growth. Some of the factors found to be responsible for decreased root growth are inhibition of root cell division by Al (Taylor, 1988), inhibition of root cell elongation by Cu and Zn (Wainwright and Woolhouse, 1977), and the extension of the cell cycle by Zn (Povell *et al.,* 1986). Metal toxicity can affect not only the length of the primary root, but also the morphology of the whole root system. In particular, toxic concentrations of Al, Pb, Cd, and Mn have been reported to enhance lateral root formation, thereby making the root system denser and more compact (Breckle, 1989). In contrast, the root hair density is generally decreased. These morphological changes may alter the root hormone balance and lower the capacity of plants to explore the soil for water and nutrients (Barcelo and Porschenrieder, 1990). Another consequence of high metal availability is browning of tap root tips and lateral roots (Foy *et al.,* 1978). The browning appears to be due to metal-stimulated suberization. Root lignification is also enhanced (Paivoke, 1983). Both suberization and lignification of roots may limit water uptake and membrane water permeability of plant cells (Breckle, 1989). This could result in a decrease of the diameter of xylem elements and water conductivity in metal-exposed plants. For example, toxic levels of Cd and Al have decreased both vessel diameter and number of vessels in *Phaseolus vulgaris* (bush bean) and *Zea mays* (maize) (Bennet *et al.,* 1985; Barcelo *et al.,* 1988). As a result of

insufficient absorption of nutrients and water from metal-damaged roots, shoot cell enlargement is also reduced. In a study of Aidid and Okamoto (1993), elongation growth rate of stem cells of *Impatiens balsamina* (balsam) was inhibited by Pb, Cd and Zn due to their suppression on both cell turgor and cell wall extensibility.

2.3.2. Mechanisms of metal uptake

One of the most important factors affecting plant accumulation of metals is their mobility and, thus, availability in soil. High concentration of heavy metals in a soil solution does not necessarily imply their high availability for plant uptake. Although it is generally assumed that plants prefer the free or uncomplexed metal ion, there are a number of soil factors controlling metal availability. Some of the factors include presence of organic matter (quantity and quality: particulate and dissolved), hydrous ion oxides, Al hydroxides, clay minerals, carbonates, phosphates, sulfates, and silicates. Adsorption and precipitation are the main reactions which are both time and pH dependent and somewhat governed by ionic strength (McBride, 1994). For example, while some metals, such as Zn and Cd, are mostly present in a soluble or exchangeable and, thus, readily bioavailable form, others, such as Pb, occur as insoluble precipitates (phosphates, carbonates, and hydroxides), which are unavailable for plant uptake (Pitchel *et al.*, 1999). However, plants have developed specific mechanisms to solubilize metals in soil, thereby enhancing their extraction.

Some plants release phytosiderophores (for example, mugenic and avenic acids) under Cu, Zn, Mn, and Fe deficiencies (Marschner, 1986; Romheld, 1991), whereas others produce thiol-containing metal binding proteins (metallothioneins) or phytochelatins (Khan *et al.*, 2000). Glutathione, being the most abundant metal-binding peptide, has been observed to be induced in Cd-exposed plants (Guo and Marschner, 1995; Salt *et al.*, 1998). In addition, plants can reduce soil-bound metal ions, including Cu and Fe, by specific plasma membrane-bound enzymes (metal reductases) (Salt *et al.*, 1998). Moreover, they can release As, Cu and Mn, but immobilize Cr by exchange with protons (Lepp, 1981). Finally, low-molecular-weight organic acids, originating from root exudates (for example, citric, oxalic, and malic acid), can also complex Cu, Pb, and Cd in the plant vacuole (Marschner, 1986). It should be noted that all of the above-mentioned processes of metal solubilization could also be performed by rhizospheric microorganisms, such as mycorrhizal fungi or root-colonizing bacteria (Salt *et al.*, 1998). For example, Davies *et al.* (2001) reported a 3-fold increase of Cr (III) accumulation in the roots and a 3.4-fold increase of Cr (VI) level in the leaves of *Helianthus annuus* (sunflower) with an arbuscular mycorrhiza genus *Glomus intraradices*. Further, Salt *et al.* (1995) illustrated 3-fold increase of Cd concentration (0.1 mg L^{-1} exposed) in the shoots of 2 week-old *B. juncea* seedlings with root-colonizing strains of *Pseudomonas putida* and *Bacillus thuringiensis*.

Once in the soil solution, metal ions can enter plant root cells either via cell walls (apoplastically) or the plasmodesmata (symplastically). The electrical charge of metal ions prevents them from moving freely across the lipophilic cellular membranes (Lasat, 2002). Therefore, most metal ions enter plant cells by an energy-dependent process via specific channels or ion carriers (transport proteins) (Clarkson and Luttge, 1989), while metal-chelated complexes can be transported via specialized carriers (Crowley *et al.*, 1991).

2.4. *Brassica juncea*

2.4.1. Geographical distribution

Brassica juncea is of Himalayan origin and is grown widely in the north portion of the Indian subcontinent and in various parts of China (Kimber and McGregor, 1995). In addition, it has been grown in the temperate regions of the world, particularly on the American Great Plains, in Hungary, and in Britain (Encyclopedia Britannica, 2003). In Canada, this crop shows high yield in the southern low-rainfall prairie areas (Mendham and Salisbury, 1995).

2.4.2. Use

The crops in the mustard family are principally used as spices. However, because of their unpalatable flavor, the seeds of *B. juncea* are unsuitable for

condiment purposes. Therefore, the *B. juncea* cultivars are mostly grown for crushing to obtain oil (Tsunoda *et al.*, 1980).

2.4.3. Life cycle

B. juncea is a fast-growing (90 to 95 days from sowing), cool-season (early spring) annual crop and seeds are harvested in early autumn (Encyclopedia Britannica, 2003). All mustards, including *B. juncea*, have four distinct phases of development. During seedling phase, the above-ground mass of the plants consists of the hypocotyl and two photosynthetic cotyledons, which in *B. juncea* are wide with deep notches (Tsunoda *et al.*, 1980). The seedling phase lasts from 7 to 10 days. The next developmental phase of mustards is vegetative growth, in which plants produce leaves for 3 to 6 weeks. *B. juncea* has simple pinnate leaves with alternate arrangement (Tsunoda *et al.*, 1980) and stems that are glaucous to bluish green in color (Hemingway, 1995). From the vegetative growth stage, the plants grow rapidly and enter a phase of dense flowering. Flowering is indeterminate and begins from the base to the top of the inflorescence. At flowering, plants bolt, opening 4 to 5 yellow flowers per day over 2 to 3 weeks. The flowers remain open for about 3-4 days before final shedding of petals (Labana and Suringer, 1984). Two of the six stamens in the flower structure are lower and shorter than others (Encyclopedia Britannica, 2003). Flowers of *B. juncea* are easily pollinated by wind and insects, however, they are about 80% self-pollinating (Labana and Suringer, 1984). The final

growth stage of mustards is fruiting. It lasts 6 to 10 weeks after sowing, and it terminates by the senescence of the sickle-shaped green pods. The seed pod usually consists of two outside walls, separated by a thin white partition (Encyclopedia Britannica, 2003). The pods of *B. juncea* contain up to 20 seeds each and the crop averages 408,000 seeds kg^{-1} (Hemingway, 1995). The mature plant can reach a maximum height of 4 feet (USDA NRCS, 2003).

2.4.4. Growth requirements

2.4.4.1. Soil type

B. juncea has many valuable characteristics that make it unique among the other members of the mustard family. It can easily grow on many different types of soils. For example, this species is well adapted to fine (clay) and medium-textured (loam) soils (Madson, 1951). Although *B. juncea* prefers neutral to slightly alkaline soil conditions, it can tolerate and grow normally at pH as low as 5.6 (Hemingway, 1995).

2.4.4.2. Soil fertility

B. juncea does not require more than normal soil levels of nitrogen (N), potassium (K) or phosphorous (P). However, in this plant, improved fertilization (60, 40, and 20 kg ha^{-1} N, P_2O_5, and K_2O, respectively) has significantly increased its growth (plant height and leaf area) and seed yield when irrigated

with saline waters of different concentrations (50, 100, and 150 meq L^{-1}) (Garg *et al.*, 1993). These authors suggested that an improvement in the concentration and uptake of N, P, and K under high fertility stimulates the activity of nitrate reductase and other ammonia assimilating enzymes. This causes the level of soluble protein and amino acids to increase greatly and contribute to the improved plant performance under salt stress.

2.4.4.3. Temperature

Studies have shown that *B. juncea* can tolerate spring frosts without serious harm (Hemingway, 1995). In contrast, this plant has shown to be sensitive to heat stress. For example, Angadi *et al.* (2000) investigated the effect of short periods of high temperature stress on the reproductive development and yield of *Brassica* species. The researchers found that high temperature treatment, i.e., 35°C, was harmful to reproductive organs at different developmental stages. In particular, it caused a decrease both in the number of seeds produced by the main stem and the number of fertile pods.

2.4.4.4. Water

B. juncea can be easily grown on land of adequate moisture supply. It is more tolerant of drought than *Brassica napus* (canola) but less than *Triticum*

aestivum (wheat). Good moisture prolongs flowering, which results in increased seed number and higher yield (Hemingway, 1995).

2.4.5. Heavy metal uptake

B. juncea has the remarkable capacity to accumulate high levels of heavy metals. For example, its shoots have been reported to take up as high as 308,600 mg Ni kg^{-1} plant (DW) and 172,300 mg Zn kg^{-1} plant (DW) in a fertilized sand/Perlite mixture supplemented with 100 mg Me kg^{-1} soil (Kumar *et al.,* 1995). *B. juncea* has also demonstrated a strong accumulation of strontium radioactive isotope (^{90}Sr), found in the soils in the Chernobyl regions of Ukraine (Salt *et al.,* 1995). In particular, the final concentration of this radionuclide in shoots of the plant was 12-fold higher than in the soil. In another experiment, the shoot Cu concentration in this plant was 31.2 mg kg^{-1} plant (DW), more than three times greater than the mean Cu concentration in the shoots of the non-treated plants, i.e., 10 mg kg^{-1} plant (DW) (Ebbs and Kochian, 1997). In addition, the roots treated with 10^{-4} M Pb accumulated a substantial amount of Pb (15,982.8 $\mu g\ g^{-1}$ plant DW), which was about 184 fold of control plants (Liu *et al.*, 2000). The authors have also reported that over 95% of the Pb accumulated in treated plants was found in the roots.

2.4.6. Heavy metal phytotoxicity

2.4.6.1. Effects on shoots

Kumar *et al.* (1995) reported that *B. juncea* showed mild chlorosis when grown for 14 days in a Zn-contaminated soil. However, there was no evidence of any toxicity of the plant grown on the same medium with 10 mg Cu kg^{-1} soil. The leaves had little decrease in chlorophyll when grown in the presence of 6.5 mg L^{-1} Zn and 0.32 mg L^{-1} Cu (Ebbs and Kochian, 1997). The same effect on chlorophyll content has been observed in *B. juncea* treated with 4mM Pb. In contrast, no reduction in chlorophyll content has been noticed in plants treated with 2 mM Ni (Burd *et al.*, 2000). Liu *et al.* (2000) have reported the reduction in the number of leaves in *B. juncea* grown on a Pb-treated soil. In particular, while control seedlings had 4 or 5 leaves, seedlings supplied with 10^{-5}, 10^{-4} and 10^{-3} M Pb had 3, 4 and 2 leaves, respectively. Similarly, Haag-Kerwer *et al.* (1999) have demonstrated inhibition in leaf expansion (the 3rd and 4th leaves) in *B. juncea* exposed to 25 μM $CdNO_3$. The same researchers have also observed the decline in transpiration rate, while the CO_2 assimilation rate (photosynthesis) of the plants had remained unaffected. Further, Begonia *et al.* (1998) found that the total leaf area of *B. juncea* treated with 250 and 500 mg L^{-1} Pb was reduced 25% and 47%, respectively, compared to the untreated plants. The same authors and Daniels-Davis (1996) have noted anthocyanin pigmentation or purplish coloration of leaves of *B. juncea* treated with 500 mg L^{-1} of Pb.

Some researchers found a significant decrease in shoot dry weight of *B. juncea* when grown on soils contaminated with heavy metals such as Pb (500 mg Pb kg^{-1} soil: Kumar *et al.*, 1995; Daniels-Davis, 1996; 10^{-3} M Pb: Liu *et al.*, 2000), and Cu (0.32 mg Cu L^{-1}: Ebbs and Kochian, 1997), while other researchers did not observe any effect of Pb (100, 250 and 500 mg Pb L^{-1}: Begonia *et al.*, 1998; 4mM Pb: Burd *et al.*, 2000), Zn (6.5 mg Zn L^{-1}: Ebbs and Kochian, 1997), Cu and Cd (10 mg Cu kg^{-1} and 2 mg Cd kg^{-1}: Kumar *et al.*, 1995) on shoot biomass of the plant. While the presence of 5 and 10 mM Zn inhibited seedling growth, Zn at 0.05 mM promoted growth of *B. juncea* seedlings, expressed in a higher shoot length of the treated plants than that of the control plants (Prasad *et al.*, 1999).

2.4.6.2. Effects on roots

Stunting or reduced root biomass is a commonly observed growth response of heavy metal-treated *B. juncea*. For instance, Ebbs and Kochian (1997) have reported a greater reduction in root dry weight in plants treated with Cu compared to those treated with Zn. In the same experiment, Cu was also shown to inhibit lateral root development. In particular, the density of lateral roots in the Cu-treated plants was significantly less than that of the control plants. In addition, a decrease in root length of *B. juncea* was observed (Liu *et al.*, 2000). Aside from stunting, a purplish color was noted on main roots in Pb-treated *B. juncea* plants in contrast to white roots of untreated plants (Begonia *et al.*, 1998).

In Cu-treated plants, primary laterals have appeared discolored with a red-brown coloration of root tips (Ebbs and Kochian, 1997).

2.5. Chromium and Plants

2.5.1. Chromium impact on plant growth and development

2.5.1.1. Beneficial effects

Chromium is not considered an essential element for plants (Tinker, 1981). Nevertheless, there are several reports on stimulation of plant growth by small concentrations of Cr. Bertrand and de Wolf (1965) have illustrated that applications of 40 g Cr ha^{-1} considerably increased yields of *Pisum sativum* (pea) and *Daucus carota* (carrot). In another study, in Russia, the addition of K_2CrO_4 (600 g ha^{-1}) has been observed to improve the weight, size, sugar content, and yield of *Vitus vinifera* (grape) (Dobrolyubskii and Slavvo, 1958). Similarly, Pratt (1966) has reported that applications of $K_2Cr_2O_7$ at 30 and 100 g kg^{-1} soil increased the yield of *Cucumis sativus* (cucumber). The reasons for the beneficial effect of a low Cr concentration on plants are not known. Some theories revolve around indirect effects of Cr on mineral nutrition, antifungal agents (Pratt, 1966), and water relations (Barcelo *et al.,* 1986).

2.5.1.2. Visible deleterious effects

The phytotoxic properties of Cr have been demonstrated in solution culture at concentrations as low as 1-2 mg L^{-1} (Baker and Brooks, 1989). Chromium is known to cause chlorosis and necrosis of leaves (Barcelo *et al.*, 1986) and to inhibit photosynthesis (Sharma *et al.*, 1995). These toxicity effects are largely concentration-dependent. For example, in *Avena sativum* (oat), when supplied with 5 and 10 mg Cr (VI), as K_2CrO_4, kg^{-1} soil, the effect was one of chlorosis, while, in the same plants, supplied with 15, 25, and 50 mg Cr (VI) kg^{-1} soil, specific symptoms of Cr toxicity appeared, including plant stunting and brownish-red coloration of leaves (Hunter and Vergnano, 1953). Gupta *et al.* (1994) found a decline in total chlorophyll content in a dose-dependent manner both in *Bacopa monnieri* (bacopa) and *Scirpus lacustris* (bulrush). Reduction in chlorophyll content in upper leaves of Cr-treated plants is thought to be due to inhibition of Fe and translocation and stimulation of Fe-deficiency symptoms (Barcelo *et al.,* 1985). The growth reduction and chlorosis may also be considered as consequences of toxic Cr effects in roots principally caused by alterations in the content of essential mineral nutrients (Barcelo *et al.*, 1985). Inhibition of root elongation has been observed in seedlings of *P. vulgaris*, treated with 96 μM Cr (VI) for 21 days (Vazquez *et al.,* 1987), and *Salvia sclarea* (cedar sage), treated with 17-34 μM Cr (VI) for 48 hours (Corradi *et al.,* 1993).

2.5.1.3. Cr (III) and Cr (VI) phytotoxicities

The prevailing view is that Cr (VI) is more toxic to plants than Cr (III). For example, Shahandeh and Hossner (2000) demonstrated that Cr (VI) application decreased the index of tolerance for *B. juncea* plants more than Cr (III) application, although it was noted that the concentration of the former in roots and shoots was greater than that of the latter. In another experiment, although both Cr (III) and Cr (VI) inhibited both root and shoot growth characteristics of *T. aestivum*, the most pronounced symptoms were observed in the roots and with Cr (VI) treatment (Mukhopadhyay and Aery, 2000). In particular, 65% reduction in shoot fresh weight was observed in plants treated with 20 mg L^{-1} of $K_2Cr_2O_7$, while about 50% reduction was found in plants supplied with the same concentration of $CrCl_3$. Moreover, in the Cr (VI)-stressed plants, the length of roots declined by 80% compared to 47% of that in the shoots (Mukhopadhyay and Aery, 2000). The observed differences in toxicity between the two forms of Cr can be explained by the low solubility and, hence, low bioavailability (more difficult penetration of cell membranes) of Cr (III) in soil (Barlett and James, 1996) as well as a greater tendency of Cr (III) to form large hydroxyl-polymers with many ligands at neutral pH level (Mukhopadhyay and Aery, 2000). On the other hand, Cr (VI) is more mobile in soils and easily penetrates cell membranes. However, if conditions are adjusted to achieve equal concentrations of Cr (III) and Cr (VI), so that both Cr forms are available to plants, toxicity may occur. For instance, chlorotic leaves and poor plant growth

have been noticed in *Brassica oleracea* (broccoli) grown for 55 days with a nutrient solution containing 10 mg kg^{-1} of Cr (III) and 1 and 10 mg kg^{-1} of Cr (VI) (Hara and Sonoda, 1979). Similarly, McGrath (1982) found that the growth of *A. sativa* seedlings was nearly ceased or inhibited when supplied with 200 μM of Cr (IIII) or 20 μM of Cr (VI) in nutrient solution. Additionally, Yamaguchi and Aso (1977) reported that 200 mg kg^{-1} of Cr (III) in soil decreased root elongation of *Oryza sativa* (rice) and *T. aestivum*, while shoots were unaffected. In yet another study, *B. oleracea* responded to excess (500 μM) supply of Cr (III) by developing a decrease in chlorophyll concentration and activity of the heme enzymes, namely catalase and peroxidase (Pandey and Sharma, 2002). The authors explained that the high affinity of Cr (III) for proteins allowed it to bind these essential enzymes, thereby inactivating them.

2.5.1.4. Morphological and anatomical alterations

Visible symptoms of metal toxicity stress appear to be a result of morphological and anatomical (at both cellular and ultracellular levels) modifications. For example, injuries of the root surface expressed by severely damaged epidermal and cortical cells, damage of root cap, collapse of root hairs and stomata and cotyledon hairs, and reduced amounts of chlorophyll and carotenoids have been observed (Vazquez *et al.,* 1987; Corradi *et al.,* 1993). The authors have assumed that, due to oxidation of cell wall and membrane components by strongly oxidizing Cr (VI), an impaired function of the plasma

membrane leading to plasmolysis may account for alterations in the content of essential mineral nutrients and water loss and explain reduced intercellular spaces, changes within chloroplasts and the wilting of root and cotyledon hairs. In addition, accumulation of abnormally high starch levels has occurred in parenchyma cells of the vascular cylinder (xylem and phloem) in the upper part of the root and in the pith of the stem, suggesting that reduced root growth due to Cr (VI) may lower starch utilization in growth processes (Vazquez *et al.,* 1987). Moreover, the occurrence of ameboid plastids in Cr (VI)-treated roots (Vazquez *et al.,* 1987) suggests that Cr (VI) may inhibit normal plastid development.

There are some reports that describe the influence of Cr (VI) toxicity on the vascular system. For example, Cr (VI) has caused a substantial decrease of the diameter of xylem vessels (Barcelo and Poschenrieder, 1990). In a similar study, the sizes of both phloem and xylem cells have decreased in stems of Cr (VI)-treated *P. vulgaris* (Vazquez *et al.,* 1987). In another study, the number of vascular bundles has increased, while the vessel density, dimension of vessel elements and number of fibers all have decreased greatly in the root and shoot of *S. lacustris*, treated with Cr (VI) (Suseela *et al.,* 2002).

Microscopic studies of leaves illustrate increased number of trichomes, but reduced number of granal stacks when treated with Cr (VI) (Barcelo *et al.,*

1988; Vazquez *et al.,* 1987). Furthermore, stomata closure and poor development of the thylakoid membrane system have been observed in Cr (VI)-treated plants. These results are hypothesized to be due to an indirect effect of Cr in leaves by induction of Fe deficiency.

2.5.2. Chromium absorption and distribution

The absorption and dynamics of Cr (III) and Cr (VI) in various plant parts have been studied in nutrient solutions (Shewry and Peterson, 1974; Skeffington *et al.*, 1976; Cary *et al.*, 1977; McGrath, 1982; Lytle *et al.*, 1998; Zayed *et al.*, 1998; Arteaga *et al.*, 2000; Davies *et al.*, 2001; Aldrich *et al.*, 2003), artificial soil mixtures (Parr and Taylor, 1980; Barcelo *et al.*, 1986; Kumar *et al.*, 1995; Suseela *et al.*, 2002), and soils (McGrath, 1982; Naqvi and Rizvi, 2000; Shahandeh and Hossner, 2000; Singh, 2001; Fernandes *et al.*, 2002). In laboratory experiments, both Cr (III) and Cr (VI) accumulate mainly in roots and are poorly translocated to shoots, although absorption and translocation of the latter is higher than that of the former (Shewry and Peterson, 1974; Barcelo *et al.*, 1986; Zayed *et al.*, 1998; Shahandeh and Hossner, 2000). A separate uptake mechanism is believed to exist for the two chromium forms. Cr (VI) is taken up actively by the sulfate carrier, while Cr (III) is absorbed passively (Shewry and Peterson, 1974; Skeffington *et al.*, 1976), being bound to the cation-exchange sites of the cell walls (Marschner, 1986). Moreover, Cr (III), chelated in soils, has been observed to enter plant roots at slower rates than

non-chelated Cr (III) and Cr (VI). Nevertheless, complexed Cr (III) moves easily to the shoots (Verfaillie, 1974). Both Cr (III) and Cr (VI) enter the vascular tissue with difficulty; however, once in the xylem, they move more freely (Skeffington *et al.*, 1976).

Uptake of Cr by plants and subsequently its accumulation in plant tissues is influenced by the amount of added metal (Barcelo *et al.*, 1985). In particular, Cr absorption has been enhanced with increasing concentration of the applied metal (Davies *et al.*, 2001; Shahandeh and Hossner, 2000; Fernandes *et al.*, 2002). In addition, probably due to the chemical similarity of the ions, the uptake of Cr (VI) is most likely to be severely inhibited by the sulfate normally present in soils (Shewry and Peterson, 1974).

According to some researchers, Cr (VI) is readily translocated within plants (Swamy, 1996; Fernandes *et al.*, 2002), while others have reported an initial reduction of Cr (VI) to mobile Cr (V) (Micera and Dessi, 1988) and to Cr (III) species (Lytle *et al.*, 1998; Zayed *et al.*, 1998; Aldrich *et al.*, 2003). For instance, in their studies with *Hordeum vulgare* (barley), Shewry and Peterson (1974) have found most of Cr in a soluble non-particulate form. They observed that two-thirds of internal Cr was located in vacuoles of root cells, while most of the remaining Cr was within the cell walls. The authors concluded that Cr is unavailable for transport mainly due to its spatial localization in a specific

subcellular compartment in the root cells i.e., the vacuole, or the lack of a specific mechanism for transport. Sanita di Toppi *et al.* (2002) further hypothesized that Cr solubilization followed by detoxification might be performed by chelation and compartmentalization in the vacuole by low-molecular-weight organic acids originating from root exudates. The X-ray absorption spectroscopy (XAS) data presented by Arteaga *et al.* (2000) also showed that Cr (VI) absorbed from solution was partially reduced to Cr (III) in the roots of *Larrea tridentate* (creosote bush). Some Cr (VI) and the reduced Cr (III) were further transported through the stems and finally accumulated as Cr (III) in the leaves of the plant, bound to oxygen-containing ligands. The form in which Cr is taken up and translocated in plants may therefore vary in different plant species.

3.0. MATERIALS AND METHODS

3.1. Soil Source and Preparation

Surface horizon (0 to 30 cm) of Orthic Humo-Ferric Podzol from Aleza Lake (Research Forests, BC, Canada) with known chemical and physical characteristics (Arocena and Sanborn, 1999) was collected (Table 3.1, Table 1: Appendix). Fresh-field soil sample was mixed thoroughly and sieved through a 4-mm polyethylene sieve before analysis in order to preserve some of soil crumb structure and aeration status of field soils during storage (Bartlett and James, 1996). Half of the soil was air-dried in a laboratory and passed through a 2-mm sieve, while the other half was stored field-moist in a refrigerator at 4°C (Fisher Scientific, Indiana, PA, USA).

The soil was analyzed for moisture content by a gravimetric method with oven-drying at 105°C (Yamato, Japan) for 24 h (Kalra and Maynard, 1991). In addition, background soil total Cr concentration was estimated by using inductively coupled plasma-atomic emission spectrometry (ICP-AES) (EPA, 1996a) following acid digestion of 0.2 g soil (EPA, 1996b). SO-2 (16 mg Cr kg^{-1} soil DW) and SO-4 (61 mg Cr kg^{-1} soil DW) Canadian certified reference soils (Canada Centre for Mineral and Energy Technology, Ottawa, ON, Canada) were used to ensure the accuracy of the measurements.

Table 3.1. Physical and chemical properties of soil from Aleza Lake.

Soil Properties	*Orthic Humo-Ferric Podzol*
Sand (weight %)*	17.9
Silt (weight %)*	67.7
Clay (< 2μm) (weight %)*	14.3
Total C (%)*	1.38
Moisture content (θm) ($kg\ kg^{-1}$)	0.12
pH in H_2O (1:2)*	5.13
CEC ($cmolc\ g^{-1}$)*	5.01
Total Cr ($mg\ kg^{-1}$)	55.3
Al_p (%)*	0.48
Fe_p (%)*	0.68

* From Arocena and Sanborn, 1999

3.2. Chromium Application

In order to ensure easy access to the roots of *Brassica juncea*, two plexiglass-made rhizotron units, covered with aluminum foil to reduce light

penetration to plant roots, were used for plant growth (Figure 3.1). Prior to filling each unit of a rhizotron with a 450 g of soil (DW), the air-dried soil samples were brought to the same water content as the field-moist ones. Upon addition of a controlled release mini-fertilizer N-P-K (18-5-9) (Scott Osmocote, Marysville, OH, USA) and aqueous Cr (III) as $CrCl_3.6H_2O$ and Cr (VI) as $K_2Cr_2O_7$ standard solutions at the rate of 100 mg Cr (III or VI) kg^{-1} soil (DW), both field-moist and air-dried soil samples were mixed thoroughly by hand and the moisture of the soils was adjusted to field capacity. Three replicates were done for each treatment.

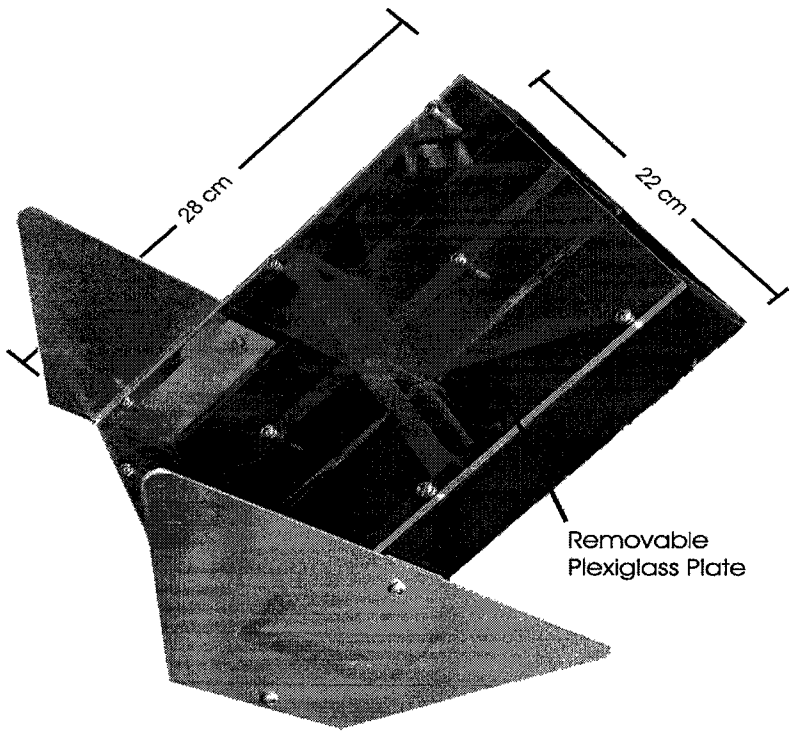

Figure 3.1. Rhizotron for growing *Brassica juncea*.

3.3. Plant Material

Seeds of *B. juncea* (accession PT 182921) were obtained from USDA/ARS Plant Introduction Station of Iowa State University (USA). Six seeds of the plant were sown in each unit of a rhizotron, previously supplied with both fertilized and Cr-treated soil.

3.4. Plant Growth and Harvest Conditions

The plants were grown in the temperature- and light-controlled environment of a greenhouse (Enhanced Forestry Laboratory, UNBC, BC, Canada) with a 16-h photoperiod and a 21/18°C day/night temperature regime. The continuous irrigation system was built with a porous polymer tube set at around field capacity water potential, i.e., -30 kPa (Brady and Weil, 2002). Deionized (reverse osmosis) water was used for irrigation. The experiment was laid out on a greenhouse bench in a blocking design (three blocks, each representing one harvest time) with a completely randomized arrangement of treatments in each rhizotron unit within each block. At one week after emergence, the plants were thinned to three seedlings within each rhizotron unit.

The plants were harvested at vegetative, flowering, and fruiting developmental stages, i.e., at 17, 36, and 69 days after sowing, respectively. At

these times, more than a half of the control and Cr-treated plants had 3 leaves emerged, visible buds, and filled seed pods, respectively.

3.5. Soil Analyses

3.5.1. Soluble Cr (III) and Cr (VI)

Total soluble Cr and Cr (VI) in the rhizosphere and bulk soil samples were extracted at each harvest time (17, 36, and 69 days after planting). Rhizosphere soil (3mm from the roots) was separated from bulk soil by gentle scooping. Cr-treated and control soil samples (1.5 g each: DW) were shaken on a reciprocating shaker (Eberbach Corp., Ann-Harbor, MI, USA) for 16 h with 15 mL of deionized water (Bolan *et al.*, 2003). All soil extracts were centrifuged at 10,000 rpm for 10 min on a Hermle Z 382 centrifuge (Mandel Scientific Comp. Ltd., Germany) and stored at 4°C (WCT Canada Inc., Cambridge, ON, Canada).

Total soluble Cr was quantified by ICP-AES on a Leeman Labs PS 1000 spectrometer (Leeman Labs Inc., USA), while Cr (VI) was determined colorimetrically at 540 nm on a Spectronic 20D+ UV spectrometer (Milton Roy Comp., USA) using a modified version of the diphenylcarbazide (DPC) procedure (Bartlett and James, 1996). The modification consisted of reducing the amount of a soil aliquot to 5 mL and the amount of the DPC reagent proportionally. Analyses were conducted in triplicates. Cr (III) was estimated

from the difference of measured values of total chromium and dichromate concentrations.

3.5.2. Microbial biomass C

At each harvest time, the Cr-treated and non-treated seedlings in field-moist and air-dried soils were gently shaken to remove the soil from the roots. The soil adhering to the roots was defined as the rhizosphere soil, while the remaining soil was bulk soil. The isolation of the rhizosphere soil was accomplished in a similar procedure as described by Priha *et al.* (1999). In particular, the roots were first washed with 35 mL of deionized water in glass tubes and sonicated mildly in a water bath (Fisher Scientific, Indiana, PA, USA) for 2 min. The solutions were centrifuged (Mandel Scientific Comp. Ltd., Germany) in 50-mL Nalgene plastic vials at 3,000 rpm for 10 min and the supernatants discarded. The roots were then immersed in another 35 mL of deionized water for further analysis of low-molecular-weight organic acids, whereas the vials with soils were weighed to determine their fresh weight and kept at 4°C (WCT Canada Inc., Cambridge, ON, Canada). For the Cr-amended and control bulk soils, 5 g of fresh soil sample was weighed into tubes with 35 mL deionized water and treated in the same manner as rhizosphere soils. After the analyses, all vials were oven-dried at 105°C (Yamato, Japan) for 24 h to determine dry weight of the soils (A&D Comp., Japan). The average dry weight of rhizosphere soil was 0.25 g.

Microbial biomass C was determined by the chloroform fumigation-extraction (CFE) method described by Priha *et al.* (1999). Both rhizosphere and bulk soil samples were fumigated with direct addition of 100 µl ethanol-free chloroform at 25°C for 24 h, whereas the non-fumigated samples were kept at 4°C (WCT Canada Inc., Cambridge, ON, Canada). After the chloroform had been removed in a vacuum dessicator (Labconco Corp., Kansas City, MO, USA) using a vacuum pump (Welch Vacuum, Thomas Industries Inc., Skokie, IL, USA), both fumigated and non-fumigated samples were extracted with 8 mL of 0.5 M K_2SO_4 for 30 min on a reciprocating shaker (Eberbach Corp., Ann-Harbor, MI, USA). The samples were then centrifuged (Mandel Scientific Company Ltd., Germany) at 1,500 rpm for 10 min and the extracts stored at -20°C in order to keep samples stable (Environmental Growth Chambers, Chagrin Falls, OH, USA) prior to the analysis. The C content of the K_2SO_4 extracts was measured by an EnviroTOC carbon analyzer (Automation Instruments Manufacturing Inc., Calgary, AB, Canada). No k_{ec}, i.e., an extractable part or fraction microbial C after fumigation, was applied; only the extractable C flush, released by fumigation (the difference between extractable C from fumigated and non-fumigated samples), was calculated.

3.5.3. Chromium speciation in soils

X-ray absorption near-edge spectroscopy (XANES) was applied to identify the oxidation state of Cr in amended rhizosphere and bulk soils. The XANES data were collected at the National Synchrotron Light Source at Brookhaven National Laboratory (NY, USA) on beam-line X18B using a silicon (Si) (111) double crystal monochromator. The Cr spectra (edge energy of 5.989 keV) were recorded using a passivated implanted planar silicon (PIPS) detector. Soil samples were packed in a sample holder with an X-ray transparent tape and placed in a sample chamber at 45° to the X-ray beam. Several spectra of two replicates were collected from 100 eV below to 100 eV above the Cr edge. Spectra were also collected for Cr (III) and Cr (VI) standard compounds, namely Cr (III)-acetate, Cr (III)-formate, Cr (III)-trioxalate, Cr (III)-chloride hexahydrated, and Cr (VI)-dichromate. Their selection was based primarily on their availability and biological significance. Standards were analyzed in a solid state. Cr reference foil was run simultaneously with each data set.

The WinXAS software package (Ressler, 2001) was used to analyze the X-ray absorption spectroscopy (XAS) collected data. The samples were calibrated against the Cr edge using the first and the second degree derivatives of the reference foil edge energy (5.989 keV). The background correction and normalization were performed on a pre-edge and a post-edge region, respectively, using a first-degree polynomial. The XANES region (5.95 to 6.05

keV) was then extracted from the entire spectra. Data were also analyzed quantitatively using linear combination (LC)-XANES fittings (Ressler, 2001) of soil samples to those of standard Cr (III) and Cr (VI) compounds.

3.6. Plant Analyses

3.6.1. Plant growth and chemical measurements

3.6.1.1. Visual evaluation of Cr stress

Visual evaluations of stress in *B. juncea*, caused by Cr, were made at the last harvest (69 days after plant sowing). The evaluation criteria included: dead plant = 100% of wilted and/or chlorotic leaves; very stressed = between 80 and 100% of wilted and/or chlorotic leaves; moderate stress = between 50% and 80% of wilted and/or chlorotic leaves; initial stress = between 20 and 50% of wilted and/or chlorotic leaves and healthy = 0% of wilted and/or chlorotic leaves.

3.6.1.2. Plant height

At the final harvest, shoot height of the control and Cr-treated *B. juncea* plants was measured from the soil surface to the shoot tip using a ruler.

3.6.1.3. Shoot and root dry weight

At each exposure period, the plants from all treatments were harvested, triple-rinsed with deionized water, and separated into roots and shoots. Their dry weights were measured (Sartorius, Germany) after oven drying at 70°C (Yamato, Japan) for 24 h (Kalra and Maynard, 1991).

3.6.1.4. Chromium accumulation

At the final harvest, the roots and shoots of Cr (III, VI)-treated and control plants were triple-rinsed with deionized water, separated into roots and shoots, which were then cut into small pieces and dried in an oven at 70°C (Yamato, Japan) for 24 h prior to the analysis. Dried plant tissues were ground with an agate mortar and a pestle and 0.25 g of the plant material was digested in concentrated HNO_3 and 30% H_2O_2 using a microwave procedure as described by Kalra and Maynard (1991). Total Cr in the digests was estimated by a Leeman Labs PS 1000 UV spectrometer (Leeman Labs Inc., USA). Standard reference material (1573a tomato leaves: 1.99 mg Cr kg^{-1} plant DW) supplied by the National Institute of Standards and Technology (Gaithersburg, MD, USA) was used to ensure accuracy of measurements.

3.6.1.5. Low-molecular-weight organic acids in root exudates

Root exudates of *B. juncea* were collected at the three sampling times. After separation of rhizosphere soil for soil microbial biomass C, the roots of both Cr (III, VI)-treated and control plants were immersed in glass tubes containing 35 mL of deionized water (Strom *et al.*, 1994). Each tube was covered with aluminum foil to avoid any possible increase in root exudation caused by light. The tubes were then randomly placed on a greenhouse bench and the plants were allowed to photosynthesize for 6 h. The light and temperature conditions were the same as for the plant growth experiment. Root exudates were collected for three plants in each replicate. Tubes with deionized water, as blanks, were treated and analyzed in the similar manner as the samples. Aqueous extracts were immediately frozen and stored at –20°C (Environmental Growth Chambers, Chagrin Falls, OH, USA) for further analysis.

Capillary electrophoresis (CE) was used to separate and consequently to identify and quantify organic acids exuded by the plant roots. The mixed standard solution was prepared from individual chemicals using nanopure (Milli-Q) water. The analyses were performed using a Beckman (Fullerton, CA, USA) model P/ACE^{TM} MDQ capillary electrophoresis system equipped with a photo diode array (PDA) detector (indirect detection operating at 233 nm) and a bare fused silica capillary column (75 µm ID x 57 cm total length). CelixirOA^{TM} pH 5.4 kit, containing pyridine-dicarboxylic acid buffering system (MicroSolv Technology

Corp., Long Branch, NJ, USA), was used to fill the capillary. To identify organic acids in root exudates, an electrokinetic (electromigration) injection of a sample (10 kV for 10 sec) was applied, while a hydrostatic (pressure) injection mode of separation (0.1 psi for 10 sec) was used for their quantification. To prepare samples for the pressure injection, 1-mL subsamples of aqueous extracts were concentrated in a freeze-dryer (LabConco Corp., Kansas City, MO, USA) overnight, just prior to the day of their analysis. Accuracy was accessed by running a mixture of standards (six times) of each acid of intermediate concentration, i.e., 100 μmol L^{-1}, within the same day as the samples.

3.6.2. Anatomical measurements

3.6.2.1. Stem anatomical characteristics

At the final harvest, a 7-cm piece of a stem from each treated and non-treated *B. juncea* was cut between the 3rd and 4th nodes from the bottom of the stem. The stem fragments were cross-sectioned manually with a double-edged razor blade and stained with a 0.1% toluidine blue (TBO) solution.

Measurements of stem diameter, width of epidermis, cortex, primary and secondary phloem and xylem, and pith were taken with a compound microscope (Olympus, Japan). The width of each plant tissue was measured in millimeters and the results were expressed in percent that a particular tissue occupied in the

plant stem. The number of xylem cells was also counted by using a compound microscope, while number of vascular bundles was determined with a dissecting microscope (Nikon, Japan).

3.6.2.2. Root and leaf anatomical characteristics

At the final harvest, a 0.5-cm segment of a tap root was taken 1 cm below the point where root and shoot joined, while a 0.5-cm segment of a lateral root was taken 0.5 cm below the point where tap and lateral root joined. In addition, a 25-mm^2 piece was cut from the center of a leaf at the 3rd node from the bottom of the stem. The root and leaf segments were processed according to the general protocol of plant preparation for microscopy (Razin, 1999). In brief, the tissues were first fixed for 3 h with 2% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2). After two rinses for 30 min and one wash overnight (at 4°C) with 0.05 M phosphate buffer, the plant samples were post-fixed for 1 h in 1% osmium tetroxide in the same buffer. The tissues were then washed with 3 changes of buffer (30 min each), dehydrated in ethanol series (25, 50, 75, 95, and 100% ethanol for 30 min each), and embedded in a medium grade LR White resin (Canemco Inc., St. Laurent, QC, Canada). Two-micron-thick longitudinal sections of the roots and cross-sections of the leaves were cut using an Ultracut E (Reichert-Jung, Austria) ultramicrotome and stained with 0.1% TBO at the University of Alberta (Edmonton, AB, Canada).

The sections were viewed under a compound microscope (Olympus 3 Max, Japan) and photographed with a Cool Pix 500 (Nikon, Japan) digital camera. All measurements were obtained using Image J software (Image Processing and Analysis in Java Software, 2003).

For lateral and tap roots, root diameter, xylem diameter, and number and width of large cells (greater or equal to 25 μm and 12.5 μm for tap and lateral roots, respectively) in the entire xylem were quantified. In addition, the number and width of all cells in a designated xylem area (200μm- and 36 μm-long for the tap and lateral roots, respectively) were determined. To choose this xylem region, first, the xylem diameter in each plant treatment was measured. The half value of the smallest xylem diameter was then used as a length of a box. Cell measurements in the tap and lateral roots were taken within this box, which was placed in the right portion of the xylem starting from its middle (Figure 3.2). For leaves, their thickness and thickness of palisade and spongy mesophyll were measured in the middle part of the leaf. The number of palisade layers and veins (vascular bundles) in the middle part of the leaf was also quantified.

3.6.2.3. Chromium speciation in plant tissues

Root and leaf samples of 69-day-old *B. juncea*, exposed to either Cr (III) or Cr (VI), were frozen, ground to a fine powder in liquid nitrogen using a pestle and a mortar and stored at -80°C in order to keep the plant tissues stable

(Sanyo, Japan). To speciate Cr, X-ray spectra of frozen plant tissues were collected at the National Synchrotron Light Source at Brookhaven National Laboratory (NY, USA) on beam-line X18B and analyzed with the WinXAS software package (Ressler, 2001) in the similar manner as the soil samples (see section 3.5.3).

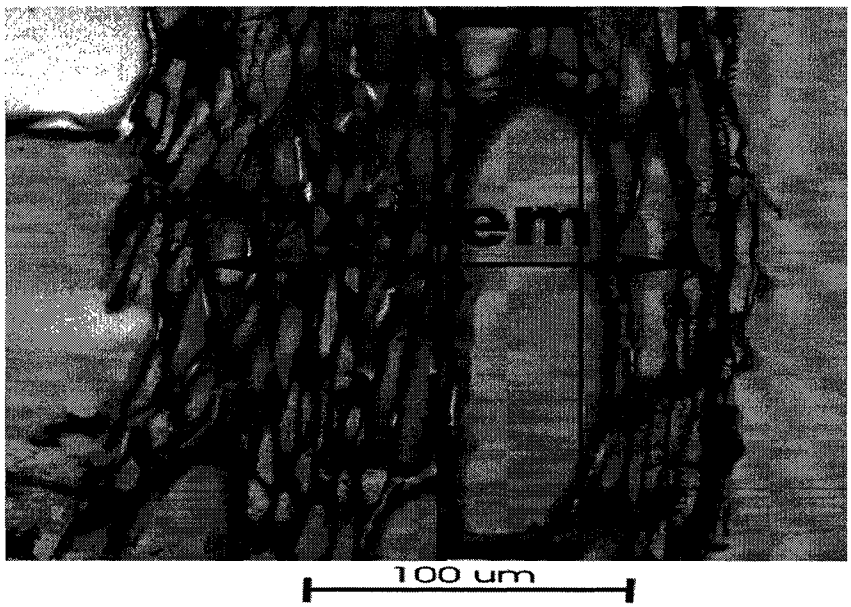

Figure 3.2. Longitudinally-sectioned lateral root (200x) from control *Brassica juncea* showingbox used for measurements of cells in a part of xylem.

3.6.2.4. Chromium localization within plant

The longitudinal sections of roots and cross-sections of leaves of Cr (III, VI)-treated *B. juncea* were prepared in the same way as for light microscopy measurements (see section 3.6.2.2).

The elemental distributions within roots and leaves were obtained using synchrotron-based X-ray microprobe spectroscopy. Measurements were performed on beamline 20-ID at the Advanced Photon Source at Argonne National Laboratory (Chicago, IL, USA). Fluorescence data were collected with a 13-element germanium (Ge) detector that was placed at 45° from the specimen and focused on a 50 μm diameter portion of the sample. The samples were scanned in 0.01 keV energy steps (5 sec per step increments). The incident photon energy was set at 5.989 keV for all scans.

3.7. Statistical Analyses

Means and standard errors were calculated to summarize the data. One-way analysis of variance (ANOVA) was performed using SPSS (Chicago, IL, USA) for Windows statistical program (SPSS, 1989-2002) in order to compare the means of all measured characteristics of Cr (III, VI)-treated and control plants. Significant differences between the means were accessed by the least significant difference (LSD) test using the General Linear Model procedure (SPSS Inc., 1989-2002).

4.0. RESULTS AND DISCUSSION

4.1. Soluble Cr (III) and Cr (VI) in soil

The data for water-soluble Cr (III) and Cr (VI) in field-moist and air-dried bulk and rhizosphere soils of *Brassica juncea*, at 17, 36, and 69 days of growth, are presented in Figure 4.1. The results indicate that generally low amounts of either Cr (III) or Cr (VI) (μg kg^{-1} levels) were extracted from soil with water, perhaps due to inefficiency of water as an extractant or rapid uptake of added Cr (III, VI) compounds by the plant.

Throughout the experiment, lower amounts of soluble Cr (III) and Cr (VI) were consistently extracted from the soils treated with 100 mg Cr (III) kg^{-1} soil than from the soils amended with the same amount of Cr (VI) (Figure 4.1). The fate of water-soluble Cr (III) compounds added to soils includes dissociation of $Cr(H_2O)_6^{3+}$ complex to the sparingly soluble Cr (III) hydroxide and protons and further formation of the even less soluble Cr (III) oxide (Grove and Ellis, 1980). The formation of protons could have decreased pH of Cr (III)-amended soils. As a result, in the acidic Aleza Lake soil used in this study (pH = 5.13: Arocena and Sanborn, 1999), this decrease in pH may have caused the precipitation of Cr (III) oxide and, thus, low initial extractability of Cr (III) by water. In contrast, dichromates are generally less likely to precipitate and are expected to be more mobile (Kimbrough *et al.*, 1999).

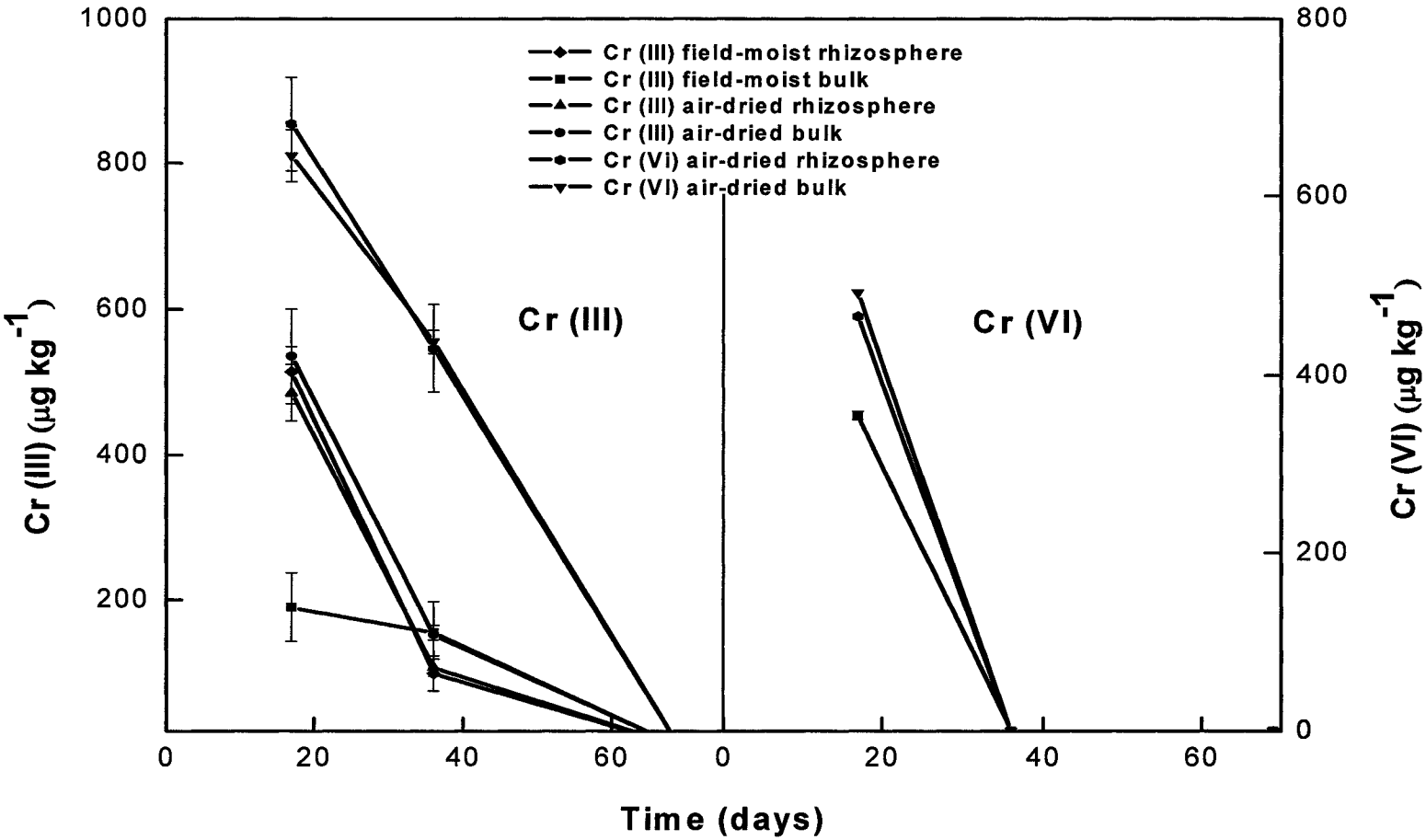

Figure 4.1. Water-soluble Cr (III) and Cr (VI) extracted from the Cr (III) and Cr (VI)-amended (100 mg kg^{-1} of either $CrCl_3 \cdot 6H_2O$ or $K_2Cr_2O_7$) field-moist and air-dried rhizosphere and bulk soils of *Brassica juncea* after 17, 36, and 69 days of growth.

Figure 4.1 also illustrates that, during the first 17 days, about 63% of water-extractable Cr in Cr (VI)-treated soils was in the form of Cr (III) species indicating the reduction of Cr (VI). Compounds with potential for reducing Cr (VI) to Cr (III) could be soil organic matter (Bartlett and Kimble, 1976) or other electron donors such as low-molecular-weight organic acids (Hale *et al.*, 1978). The latter can be formed during decomposition of organic residues in soils derived from decaying animal and microbial tissues, or released by plant root exudates (Hale *et al.*, 1978). Root exudates have been reported to affect the redox behavior of Cr because of their ability to reduce Cr (VI) and to form soluble complexes with Cr (III) species (Lundstrom, 1993). For example, organic acids such as citric, malic and aspartic acids released in root exudates in *Z. mays*, treated with different levels of Cr (III) and Cr (VI), have been reported to enrich Cr uptake possibly by the formation of organically-bound Cr (III) compounds (Srivastava *et al.*, 1998a).

During the next 19 days of metal exposure, Cr (VI) was not detected (< 24 μg kg^{-1}) in any soil and Cr treatments (Figure 4.1), suggesting that the reduction of Cr (VI) to Cr (III) in the soils could have occurred. Soil pH, bioactivity, and oxygen status are believed to be among important characteristics in accessing the reducing power of the soil (Losi *et al.*, 1994). Organic materials (total C = 1.38%: Arocena and Sanborn, 1999) appear to be limited in the Aleza Lake soil; therefore, plant root and microbial respiration (an

oxygen-consuming and carbon dioxide-releasing process) could have lowered the O_2 level, decreased pH, and provided additional C enrichment (through root exudation and decay), thereby favoring Cr (VI) reduction.

At the final harvest (69 days after plant sowing), in any soil and Cr treatment, however, no water soluble Cr (III) could be detected (< 72 μg kg^{-1}) (Figure 4.1). Therefore, a significant amount of soluble Cr (III) may have hydrolyzed, adsorbed and, thus, become unavailable.

As observed in Figure 4.1, after 17 days of metal exposure, in field-moist soils treated with Cr (III), the concentration of water-extractable Cr (III) was significantly lower, whereas the concentration of water-extractable Cr (VI) was significantly higher in the bulk compared to the rhizosphere soil. This trend was not observed in the case of either air-dry soils amended with Cr (III, VI) or field-moist soils amended with Cr (VI). Oxidation of a small portion of soluble Cr (III) added to soils, most likely by MnO_2, could possibly have contributed to the presence of soluble Cr (VI) in field-moist bulk soil and, thus, to the decrease of soluble Cr (III) in the same soil. In contrast, the most obvious effects of drying are greater solubility and reducing ability of soil organic matter (Bartlett and James, 1988). As a result, surface acidity increases and Mn (IV) is reduced to Mn (II), becoming exchangeable and soluble. Both lower pH and higher solubility of organic matter probably result from the increased polarity of surface-oriented

water as drying proceeds (Bartlett and James, 1988). Buildup of reduced Mn (II), attracted by the negative charge on the oxide surface, leads to slowing of the oxidation of Cr (III), because the adsorption of reduced Mn (II) has resulted in a plus-charged oxide surface that repels ionic Cr (III) (Bartlett, 1991). This could explain the fact that the dried-rewet soil samples did not oxidize any Cr (III) throughout the present study. In a similar study of Barlett and James (1979), although only about 7% of added Cr (III) was oxidized to Cr (VI), 10- and 5-times more Cr (VI) was formed in the moist samples than in the dried-rewet ones, after 40 hours and 15 days of Cr (III) application, respectively. On the other hand, the absence of soluble Cr (VI) in the field-moist rhizosphere soil of Cr (III)-treated plants in the present experiment (Figure 4.1) demonstrates that the rhizosphere may have prevented oxidation of Cr (III) or readily reduced formed Cr (VI) to Cr (III).

4.2. Chromium speciation in soil

Information about valence state of Cr in amended soils was obtained using XANES. XANES spectra of the reference compounds of Cr (VI) as potassium dichromate and Cr (III) as Cr (III)-chloride hexahydrated, Cr (III)-trioxalate, Cr (III)-acetate, and Cr (III)-formate are shown in Figure 4.2. As seen from this figure, the spectra of Cr (VI) and Cr (III) compounds are very different.

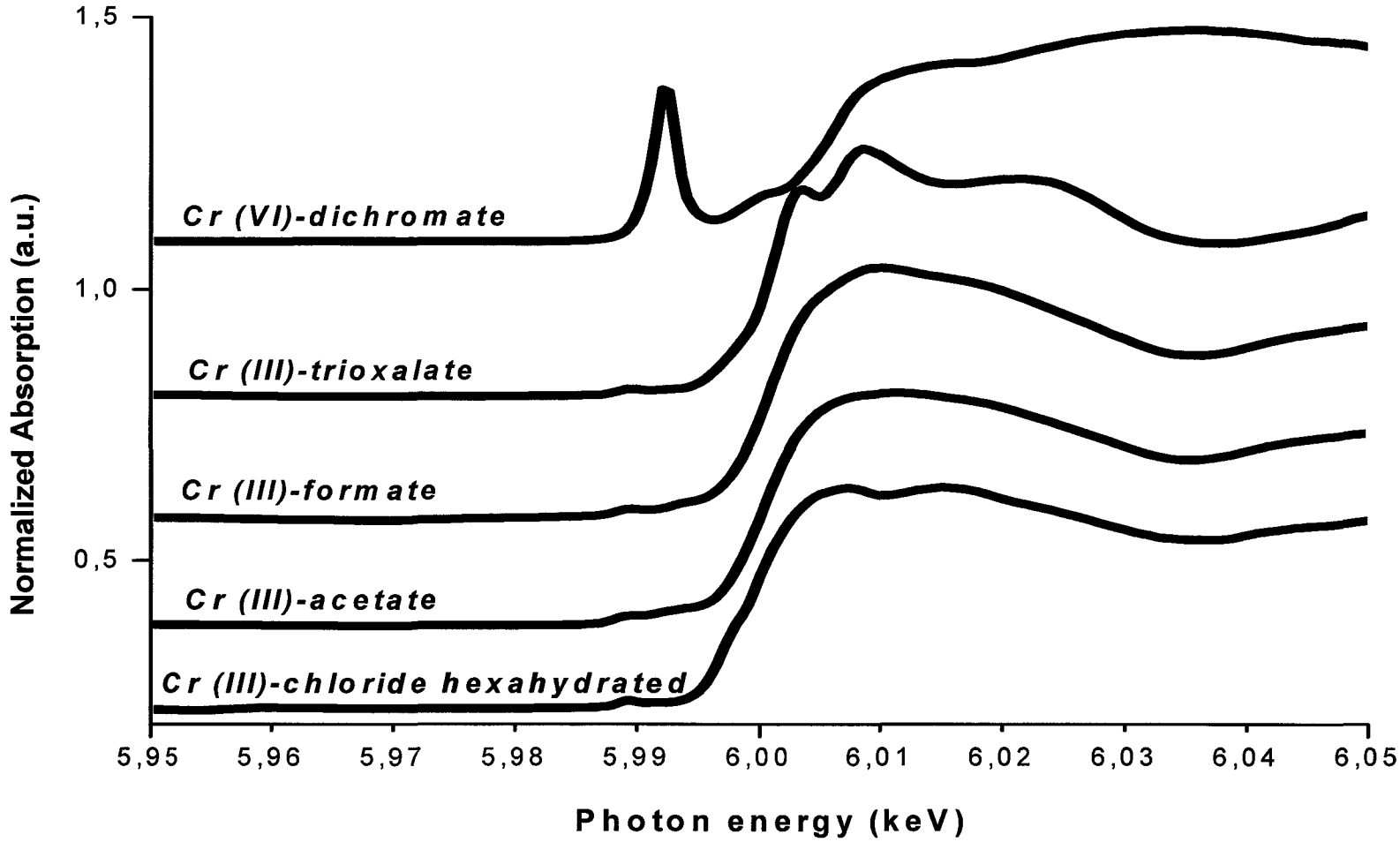

Figure 4.2. X-ray absorption near-edge spectroscopy (XANES) spectra of Cr (III, VI) reference compounds.

In particular, the pre-edge peak in Cr (VI) spectrum is intense but it is small and indistinct in all Cr (III) species. In all soil and Cr treatments, the appearance of the XANES of the rhizosphere and bulk soil samples (Figures 4.3 and 4.4) is similar to that of model Cr (III) species (Figure 4.2). It is evident, therefore, that Cr (III) is the valence state of Cr in all soils. These results are in agreement with the data on water-soluble Cr (VI), which was not detected in any soil treatments at the final plant harvest (Figure 4.1). Therefore, the reduction of Cr (VI) to Cr (III), perhaps mediated by soil organic matter or products of its oxidative degradation (non-humic organic acids) in the bulk soils and products of plant and/or microbial metabolism (exudates) in the rhizosphere soils, seems to be the major mechanism of Cr (VI) removal from soils.

The quantitative data for the linear combination (LC)-XANES fittings (Table 4.1) further support the above conclusion. None of the soils appeared to contain any potassium dichromate. In contrast, from the reference compounds used in this study, Cr (III)-formate and Cr (III)-acetate were the best fits for the bulk and rhizosphere soils, respectively, with the exception of Cr (III)-trioxalate (74%) being predominant in the Cr (III)-amended air-dried rhizophere soil (Table 4.1). These findings suggest that low-molecular-weight organic acids, particularly formic and acetic acids, could have contributed to Cr (VI) reduction.

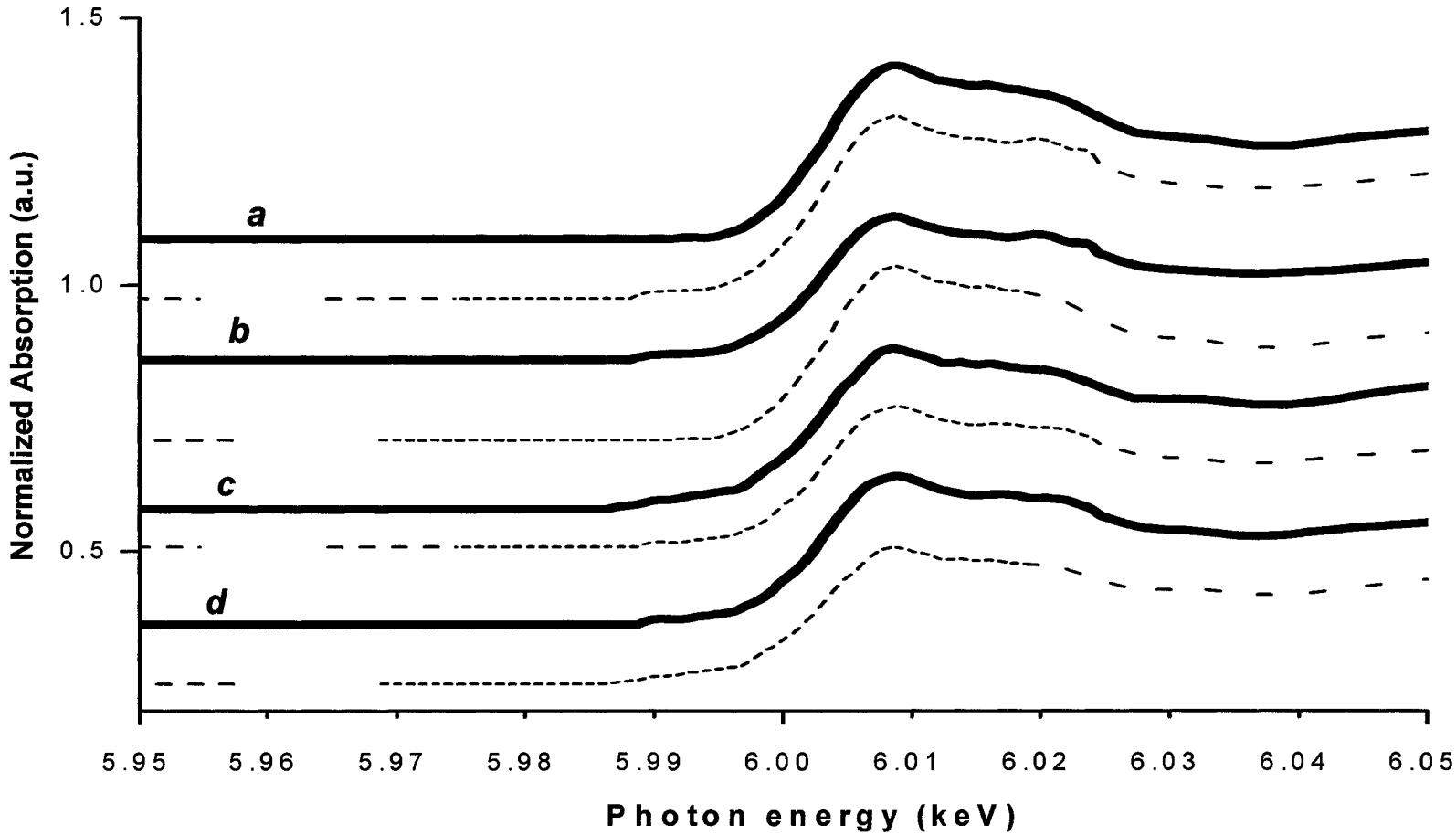

Figure 4.3. X-ray absorption near-edge spectroscopy (XANES) spectra of field-moist: (a, b) Cr (III)-amended rhizosphere and bulk soils; (c, d) Cr (VI)-amended rhizosphere and bulk soils. Dashed curves represent linear combination (LC)-XANES data fittings.

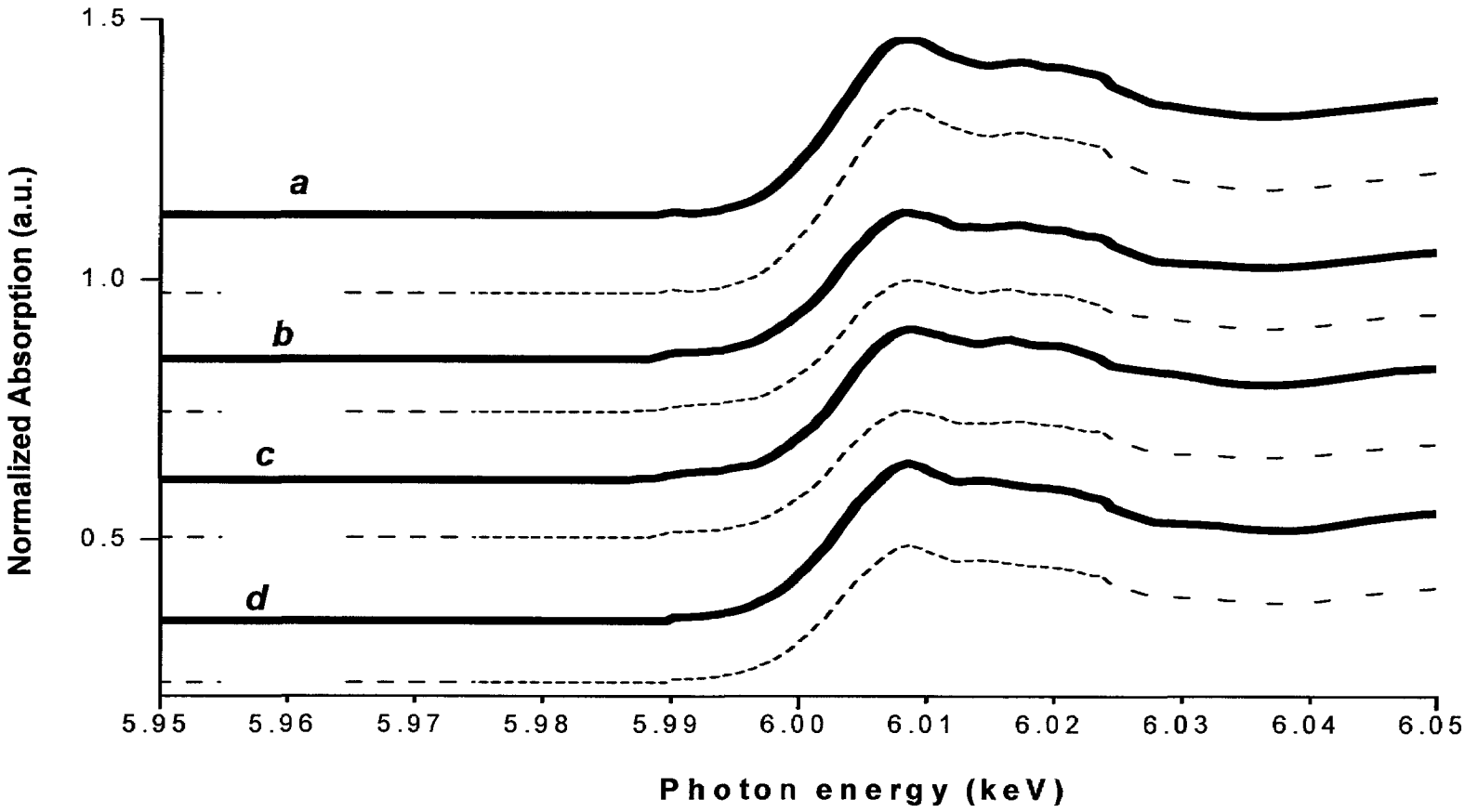

Figure 4.4. X-ray absorption near-edge spectroscopy (XANES) spectra of air-dried: (a, b) Cr (III)-amended rhizosphere and bulk soils; (c, d) Cr (VI)-amended rhizosphere and bulk soils. Dashed curves represent linear combination (LC)-XANES data fittings.

Table 4.1. Distribution of chromium compounds (%) in the rhizosphere and bulk field-moist and air-dried soils of *Brassica juncea* treated with 100 mg kg^{-1} of either $CrCl_3 \cdot 6H_2O$ or $K_2Cr_2O_7$ (n = 2).

Reference compound	***Cr (III)-amended soil***				***Cr (VI)-amended soii***			
	Field-moist		***Air-dried***		***Field-moist***		***Air-dried***	
	Rhizo-sphere	*Bulk*	*Rhizo-sphere*	*Bulk*	*Rhizo-sphere*	*Bulk*	*Rhizo-sphere*	*Bulk*
Cr (VI)-dichromate	0	0	0	0	0	0	0.0	0
Cr (III)-chloride hexahydrated	2	0	0	0	0	0	10	17
Cr (III)-formate	19	98	0	66	0	70	15	78
Cr (III)-acetate	79	2	26	18	79	30	75	5
Cr (III)-trioxalate	0	0	74	16	21	0	0	0

Being the simplest organic acid, formic acid in plants can be a product of photorespiration, fermentation, and possibly direct CO_2 reduction in chloroplasts (Igamberdiev *et al.*, 1999). In addition, it can be produced during soil organic matter decomposition (Stevenson, 1967). Moreover, soil bacteria may produce formic acid by utilizing oxalate as a C source in a series of reactions involving coenzyme A (CoA) (Hodgkinson, 1977). Acetic acid can also be formed in higher plants in an irreversible reaction of pyruvic decarboxylation metabolic pathway and during microbial fermentation (Robinson, 1986). Most low-molecular-weight organic acids are water-soluble. They can release protons and the anionic forms can function as ligands, which through surface and in-solution complexation reactions affect metals solubility and speciation (Harter and Naidu, 1995). Monovalent organic acids, including formic and acetic acids, are weakly adsorbed to the soil's solid phase (Jones *et al.*, 2003), thereby making them potential chelates of reduced Cr (III).

4.3. Chromium influence on soil microbial activity

The values of the C flush for the Cr (III, VI)-treated and control field-moist and air-dried soils are presented in Table 4.2. The C flush was generally higher ($p < 0.05$) for the rhizosphere than for the bulk soil in all treatments. To quantify the rhizosphere effect, an R/E, i.e., rhizosphere over edaphosphere or bulk soil, ratio can be used. The R/E ratio is determined by dividing the activity of

Table 4.2. Chloroform fumigation-extraction C flush (mg C kg^{-1} DW soil) in field-moist and air-dried bulk and rhizosphere soils of *Brassica juncea* treated with 100 mg kg^{-1} of either $CrCl_3.6H_2O$ or $K_2Cr_2O_7$.

	17 days				**36 days**				**69 days**			
	Field-moist		***Air-dry***		***Field-moist***		***Air-dry***		***Field-moist***		***Air-dry***	
Treatment	*Rhizo-sphere*	*Bulk*	*Rhizo-sphere*	*Bulk*	*Rhizo-sphere*	*Bulk*	*Rhizo-sphere*	*Bulk*	*Rhizo-sphere*	*Bulk*	*Rhizo-sphere*	*Bulk*
Control	22^a (1.1)[1]	3^b (0.6)	22^a (1.2)	2^b (0.2)	44^a (2)	4^b (1.2)	44^a (2)	4^b (0.11)	57^a (0.5)	6^b (0.2)	57^a (2)	6^b (0.4)
Cr (III)	22^a (0.3)	3^b (0.3)	22^a (0.3)	2^b (0.08)	42^a (1.3)	4^b (0.13)	41^a (0.4)	4^b (0.3)	57^a (3)	6^b (0.6)	56^a (2)	6^b (0.7)
Cr (VI)	18^c (0.6)	0.7^d (0.08)	17^c (0.6)	0.5^d (0.11)	35^c (1.2)	3^d (0.2)	34^c (2)	2^d (0.2)	45^c (2)	4^d (0.3)	46^c (0.5)	3^d (0.2)
F ratio and (p value)												
treatment	*5.22 (0.049)*[2]	*5.53 (0.044)*	*5.94 (0.038)*	*12.52 (0.007)*	*7.74 (0.022)*	*22.76 (0.002)*	*9.18 (0.015)*	*14.83 (0.005)*	*6.48 (0.032)*	*5.24 (0.048)*	*14.74 (0.005)*	*5.31 (0.047)*
rhizosphere x bulk												
Treatment	***Field-moist***		***Air-dry***		***Field-moist***		***Air-dry***		***Fieid-moist***		***Air-dry***	
Control	*175.19 (0.000)*		*180.52 (0.000)*		*441.71 (0.000)*		*430.27 (0.000)*		*5849.61 (0.000)*		*674.73 (0.000)*	
Cr (III)	*1142.33 (0.000)*		*2132.02 (0.000)*		*560.42 (0.000)*		*4165.47 (0.000)*		*191.02 (0.000)*		*646.43 (0.000)*	
Cr (VI)	*651.23 (0.000)*		*536.08 (0.000)*		*449.98 (0.000)*		*237.88 (0.000)*		*237.30 (0.000)*		*4858.00 (0.000)*	
field-moist x air-dry												
Treatment	***Rhizosphere***		***Bulk***		***Rhizosphere***		***Bulk***		***Rhizosphere***		***Bulk***	
Control	*0.00 (0.991)*		*1.01 (0.372)*		*0.04 (0.843)*		*1.12 (0.350)*		*0.01 (0.945)*		*0.68 (0.457)*	
Cr (III)	*0.27 (0.631)*		*0.08 (0.851)*		*0.27 (0.631)*		*1.70 (0.262)*		*0.07 (0.801)*		*0.11 (0.756)*	
Cr (VI)	*0.60 (0.483)*		*1.24 (0.327)*		*0.22 (0.666)*		*3.78 (0.124)*		*0.09 (0.780)*		*2.86 (0.166)*	

[1] Standard errors. [2] p value. No kec correction. Within each time, means followed by a common letter are not significantly different from each other using one-way ANOVA and LSD test ($\alpha = 0.05$, $n = 3$).

microorganisms (C flush in a gram of the rhizosphere soil) by the C flush in a gram of the bulk soil. In the present study, the R/E ratios varied from 8.32 to 34.92. In the rhizosphere, there is a continuous flow of organic substrates derived from roots including exudates, leaked and secreted chemicals, sloughed root cells, and mucilages which can be readily used as nutrients by microorganisms (Wardle, 1992; Curl and Truelove, 1996). As a result, microbial biomass and activity are generally higher in rhizosphere than in bulk soil. Jensen and Sorensen (1994) also found that in the *H. vulgare* rhizosphere, the SIR (substrate-induced respiration) rates were 72-170% higher than those in the bulk soil. In another study, Priha *et al.* (1999) observed increase in flushes of C and N in rhizosphere soils of *Pinus sylvestris* (Scots pine), *Picea abies* (Norway spruce) and *Betula pendula* (silver birch) as compared to the flushes measured in bulk soils.

At any Cr exposure period, in both rhizosphere and bulk soils, there was a significant decrease in the C flush due to Cr (VI) contamination, while there was no significant change ($p > 0.05$) observed in the soils treated with Cr (III) compared to those of the control (Table 4.2). Moreover, the extent of Cr (VI) inhibition did not decline with time, indicating that a permanent damage of microorganisms might have occurred. Cr (VI) appeared to be toxic to microorganisms perhaps due to its higher availability and hence, biological activity in soil (Ross *et al.*, 1981; Bartlett and James, 1988). Reports on Cr

toxicity to soil microorganisms indicate that Cr (III) is not considered to be particularly harmful (Ross *et al.*, 1981; Doelman and Haanstra, 1984, 1986; Yadav *et al.*, 1986), while Cr (VI) is shown to strongly inhibit most of biological properties such as enzyme activities, basal respiration, microbial biomass C, and denitrification (Ross *et al.*, 1981; Speir *et al.*, 1995). Despite the fact that water-extractable Cr (VI) declined markedly with time (Figure 4.1), the long-term inhibition of microorganisms caused by this form of Cr could possibly be due to a permanent damage of soil microbial population (Ross *et al.*, 1981).

Table 4.2 also shows that, during any period of Cr exposure, the estimates of C flush decreased in the bulk soils which were air-dried and then rewetted prior to Cr application compared to those which were used as field-moist. However, these changes were not significantly different ($p > 0.05$) in any treatments. Although microorganisms can be killed during desiccation (Sorensen, 1983), 70-87% of microbial biomass may eventually recover after soils are remoistened mainly due to the decomposition of various sources of organic matter that the air-drying process made available (Shan-Min *et al.*, 1987). In this study, on average, the C flush recovery was 81% (Table 4.2).

4.4. Macroscopic effects of chromium on plant growth

4.4.1. Chromium uptake

Chromium concentration in shoots and roots of *B. juncea* in the field-moist and air-dried soils, treated with either Cr (III) or Cr (VI), is shown in Table 4.3. There were no significant differences ($p < 0.05$) in either root or shoot concentration in Cr (III)-treated plants grown in field-moist and air-dried soils. This could be due to the fact that only a small portion of Cr (III) oxidized to Cr (VI) (Figure 4.1), thereby not affecting Cr concentration in plant tissues (Table 4.3).

In all soils, Cr concentration in the roots was 18-40 times higher than in the shoots (Table 4.3). In addition, the plants accumulated more Cr from the field-moist or air-dried soils supplied with Cr (VI) than those treated with Cr (III). The Cr concentration in the plant tissues, however, does not take plant biomass into consideration and, thus, might not be an accurate evaluation of the ability of *B. juncea* to extract Cr from the soil. Therefore, the total amount of Cr taken up by the roots and transported to the plant shoots was also calculated (Table 4.4). If the plant biomass is taken into account, during the growth period, the Cr (III)-treated plants, grown in the field-moist and air-dried soils, removed an average of 49 and 47.7 µg Cr per plant (148 and 143 µg or 0.3% per rhizotron),

Table 4.3. Chromium concentration in roots and shoots of *Brassica juncea* after 69 days of growth in Cr (III, VI)-contaminated field-moist and air-dried soils.

Treatment	*Roots (mg Cr kg^{-1} DW)*	*BCF*[1]	*Shoots (mg Cr kg^{-1} DW)*	*BCF*	*Root/Shoot ratio*	*Index of tolerance*[2] *(%)*
Control moist	BDL[3a]	-	2.4[a] (0.53)	-	-	-
Control dry	BDL[a]	-	2.9[a] (0.32)	-	-	-
Cr (III) moist	208[b] (5.5)	2.1	5.9[b] (0.16)	0.059	35	88
Cr (III) dry	200[b] (5.1)[4]	2.0	5.6[b] (0.33)	0.056	36	87
Cr (VI) moist	390[c] (7.1)	3.9	20[c] (1.05)	0.20	19	70
Cr (VI) dry	388[c] (7.6)	3.9	21[c] (1.43)	0.21	18	68
F ratio and (p value)	*34.57 (0.000)*[5]	-	*81.84 (0.000)*	-	-	-

[1] Bioconcentration factor = Cr (III, VI) concentration in plant tissue (mg kg^{-1}) at harvest / concentration of added Cr (III, VI) in soil (mg kg^{-1}).

[2] Index of tolerance (%) = (total DW of Cr (III), (VI)-treated plant / total DW of control plant) x 100.

[3] Below detection limit (< 4.5 μg Cr kg^{-1} DW plant).

[4] Standard error. [5] p value. Within each column, means followed by a common letter are not significantly different from each other using one-way ANOVA and LSD test (α = 0.05, n = 3).

Table 4.4. Chromium accumulation[1] in roots and shoots of *Brassica juncea* after 69 days of growth in Cr (III, VI)-contaminated field-moist and air-dried soils.

Treatment	*Root uptake (μg Cr plant^{-1})*	*Shoot uptake (μg Cr plant^{-1})*	*Total root and shoot uptake (μg Cr rhizotron^{-1})*
Control moist	BDL[2a]	2.2[a] (0.48)	6.6
Control dry	BDL[a]	2.7[a] (0.29)	8.0
Cr (III) moist	45[b] (1.4)[3]	4.0[b] (0.13)	148
Cr (III) dry	44[b] (1.3)	3.7[b] (0.21)	143
Cr (VI) moist	47[b] (0.9)	10[c] (0.52)	173
Cr (VI) dry	48[b] (1.0)	11[c] (0.77)	180
F ratio and (p value)	*378.74 (0.000)*[4]	*49.60 (0.000)*	-

[1]Root (shoot) concentration ($\mu g\ g^{-1}$) x root (shoot) DW (g).

[2]Below detection limit (< 4.5 μg Cr kg^{-1} DW plant).

[3] Standard error. [4] p value.

Within each column, means followed by a common letter are not significantly different from each other using one-way ANOVA and LSD test ($\alpha = 0.05$, $n = 3$).

respectively, while Cr (VI)-treated plants, grown in the same soil treatments, removed 57 and 59 µg Cr per plant (173 and 180 µg or 0.4% per rhizotron). Although the Cr (VI)-treated plants had a higher Cr concentration in their roots (Table 4.3), the Cr (III)-treated plants accumulated the similar amount of Cr (Table 4.4), due to their greater biomass (Figure 4.6). On the contrary, the values for the shoot accumulation were significantly higher for the Cr (VI)-treated plants compared to those of the Cr (III)-treated plants, thereby confirming greater Cr translocation in the Cr (VI) treatment. The tendency of Cr (III) to be retained on the cation-exchange sites of the root cell walls (Marschner, 1986) might explain why this Cr form was less available to the plant shoots. The increased translocation in Cr (VI)-treated *B. juncea* observed in this study might be due to damaged plant root membranes and transport of this form of Cr by simple diffusion. These results are in agreement with previous studies on Cr accumulation in diverse plant species (Cary *et al.*, 1977; Wallace *et al.*, 1977; Parr and Taylor, 1980; Shahandeh and Hossner, 2000; Davies *et al.*, 2001).

The root to shoot Cr concentration ratio was 35 or 36 and 19 or 18 for the Cr (III) or Cr (VI)-amended plants, grown in the field-moist or air-dried soils, respectively (Table 4.3). This indicates that very little Cr was translocated to the shoots, although greater translocation with Cr (VI) compared to that with Cr (III) was observed (Table 4.3). Cr (VI) has probably been reduced in the plant roots to the less biologically active Cr (III), thereby limiting Cr movement to the shoots.

Lytle *et al.* (1998) also observed Cr (VI) reduction in the fine lateral roots of *Eichhornia crassipes* (water hyacinth) after 4 hours of metal exposure. Further, Shewry and Peterson (1974) hypothesized that Cr is unavailable for transport probably due to its spatial localization in a specific subcellular compartment in the root cells (the vacuole), as a tolerance mechanism and a common feature of metal-stressed plants (McGrath *et al.*, 2002). Vazquez *et al.* (1994) observed preferential accumulation of Zn in the vacuoles of root and leaf epidermal cells of *Thlaspi caerulescens* (alpine pennycress). The Ni hyperaccumulator, *Thlaspi goesingense* (Austrian mustard), was also found to sequester Ni in the vacuoles in leaf cells (Kramer *et al.*, 2000).

Plant dry weight was used to calculate an index of Cr tolerance in each soil and Cr treatment (Table 4.3). For the plants in any of the Cr and soil treatments, the index of tolerance was greater than 50%, which is generally considered to be the minimum desired biomass production for the plants growing in a metal- contaminated site (Chang *et al.*, 1992; Baker *et al.*, 1994b). Tolerance of Cr was influenced by the form of applied Cr, but not the soil treatment (Table 4.3). In particular, plants had the index of tolerance of 87 and 88% when grown in the dry and moist soils contaminated with Cr (III), while the index of tolerance dropped to 68 and 70% in the same soils treated with Cr (VI) (Table 4.3).

The bioaccumulation (or transfer) factors (BCF) were also computed for roots and shoots in relation to Cr applied to the soils (Table 4.3). In general, the higher the BCF, the higher the plant uptake of Cr from soil. Plant roots had higher bioaccumulation factors than shoots. For the roots, the bioaccumulation factors varied from 2.0 and 2.1 to 3.9 in plants grown in field-moist and air-dried Cr (III) and Cr (VI)-treated soils, respectively (Table 4.3). For the shoots, the highest plant uptake was observed from both field-moist and air-dried Cr (VI)-treated soils as compared to those of Cr (III) (Table 4.3). *B. juncea* has been reported to accumulate Cr (Raskin *et al.*, 1994; Salt *et al.*, 1995). However, most of the studies on Cr accumulation have been performed either hydroponically or in soils contaminated with high concentrations of anthropogenic or applied Cr. For example, in an experiment of Kumar *et al.* (1995), *B. juncea* had the lowest bioaccumulation factor of 0.1 in Cr (III) treatment and the highest bioaccumulation factor of 64 in Cr (VI) treatment. Salt *et al.* (1995) also found that hydroponically grown *B. juncea* has accumulated Cr mainly in the roots with the ratio of bioaccumulation factor in roots to shoots of 70. Although *B. juncea* plants could accumulate large amounts of Cr in their roots or shoots, they died within a few days following exposure to 500 mg Cr (VI) kg^{-1} soil (Shahanden and Hossner, 2000). Given that Cr added to soil can show several fates including oxidation, reduction, adsorption, chelation, precipitation as well as remaining in the solution (Adriano, 1986; Fendorf, 1995), in the present study, solubilization could be a major problem for *B. juncea* to accumulate high Cr concentrations.

4.4.2. Chromium influence on plant visible stress

The Cr (VI) treatment resulted in the greatest visible plant stress (Table 4.5). In particular, in both moist and dry soils, some initial stress (between 20 and 50% chlorotic leaves) and moderate stress (between 50% and 80% chlorotic leaves) were noticed in 78% and 22% of Cr (VI)-treated plants, respectively. In contrast, in the Cr (III)-amended moist and air-dried soils, only 33% and 22% of the plants showed some chlorosis, respectively, while none of them experienced moderate metal stress. The literature on other plants illustrates that the effect of Cr (VI) is often more pronounced than that of Cr (III) (Satayakala and Kaiser, 1993; Jain and Aery, 1998; Mukhopadnyay and Aery, 2000). In particular, chlorosis caused by Cr (VI) was observed by many researchers (Hara and Sonoda, 1979; Vazquez *et al.*, 1987; Davies *et al.*, 2001), which was also found in the present study (Table 4.5). Chlorosis noted in the Cr (VI)-treated leaves of *B. juncea* may be a consequence of toxic effects of Cr (VI) on the plant roots and stem (Tables 4.8 and 4.10; Figures 4.6, 4.9, and 4.10). This result is consistent with past studies (Hewitt, 1948; Sharma *et al.*, 1995; Jain *et al.*, 2000). However, probably because only a small amount of Cr reached the shoots (Tables 4.3 and 4.4), mild chlorosis was observed (Table 4.5).

Table 4.5. Visible stress in *Brassica juncea* after 69 days of growth in Cr (III, VI)-contaminated field-moist and air-dried soils.

Treatment	*mg Cr kg^{-1} soil*	VISIBLE STRESS (%)				
		Dead	*Very stressed*	*Moderate stress*	*Initial stress*	*Healthy*
Control moist	0	0	0	0^a	0^a	100^a
Control dry	0	0	0	0^a	0^a	100^a
Cr (III) moist	100	0	0	0^a	33^b	67^b
Cr (III) dry	100	0	0	0^a	22^b	78^b
Cr (VI) moist	100	0	0	22^b	78^c	0^c
Cr (VI) dry	100	0	0	22^b	78^c	0^c
F ratio and (p value)	-	-	-	*8.00 (0.015)*[1]	*20.34 (0.000)*	*104.22 (0.000)*

[1] p value.

Within each column, means followed by a common letter are not significantly different from each other using one-way ANOVA and LSD test ($\alpha = 0.05$, $n = 3$).

Cr (VI) is also known to alter the content of essential mineral nutrients, including Fe (Barcelo *et al.*, 1985), which results in chlorosis. The explanation for this is that there may be competition between the toxic metal ions and Fe in enzyme systems involved in chlorophyll formation (Hewitt, 1948). In particular, the similarity of the ionic radii of Cr (III) and Fe (III) can lead to Cr (III) substitution for Fe (III) in the proteins, namely catalase and peroxidase, resulting in loss of their efficiency (Pandey and Sharma, 2002). The first appearance of chlorosis in the younger leaves of *B. juncea* may be due to general immobility of Fe within plants. As a result, the chlorosis mainly affects new growth since even healthy plants cannot take Fe from older leaves and send it to younger leaves (Jones, 1998).

4.4.3. Chromium influence on plant root and shoot growth

Plant height was not significantly affected by all Cr and soil treatments (Figure 4.5). Cell elongation is a complex process involving turgor requirements, synthesis of wall constituents, and plant growth regulators (Wainwright and Woolhouse, 1977), namely ethylene, abscisic acid, and gibberellin (Kende *et al.*, 1998). Reduced plant height caused by heavy metals, including Cr, has

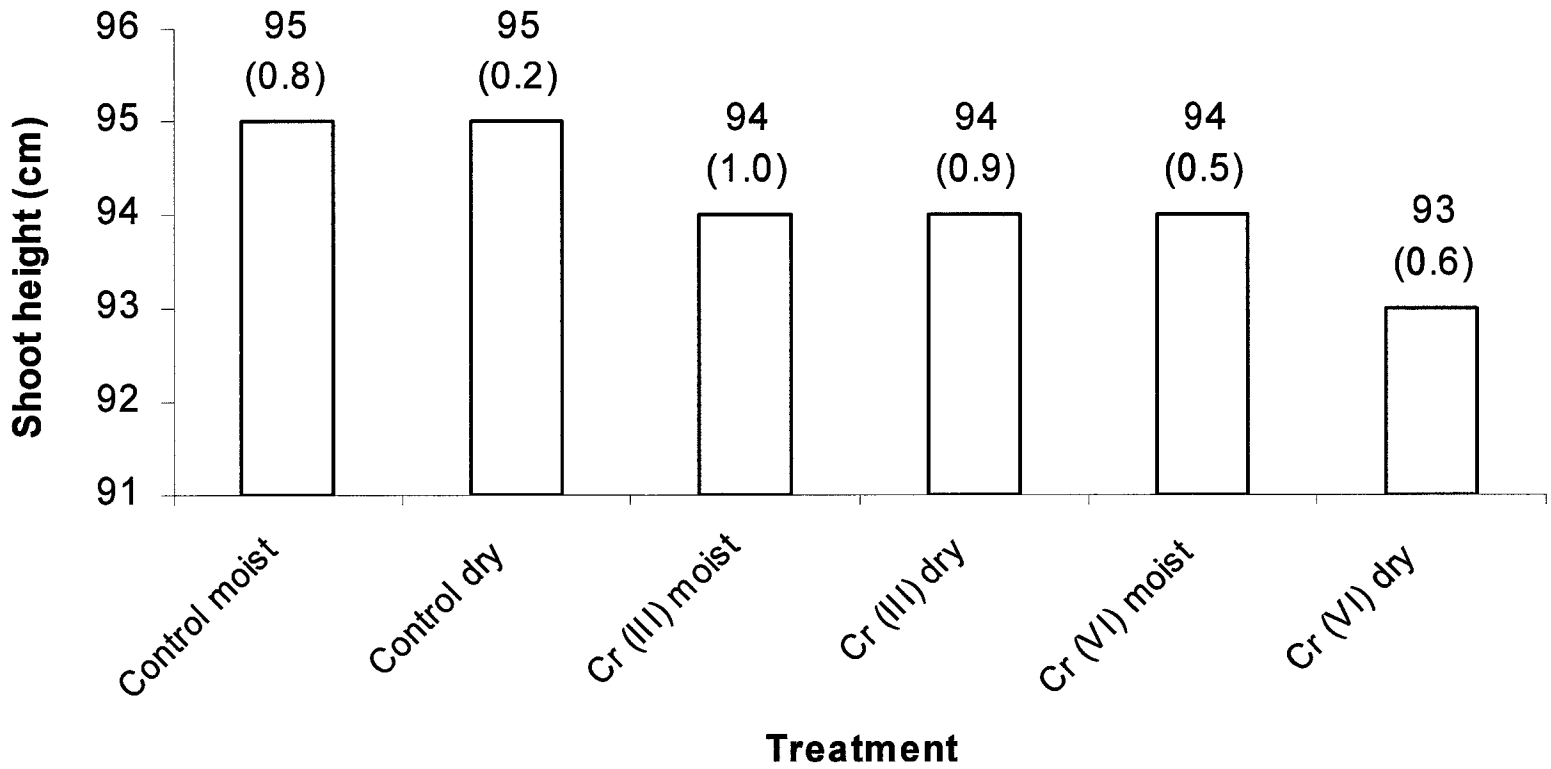

Figure 4.5. Effect of two chromium species [Cr (III) and Cr (VI)] on shoot height of *Brassica juncea* grown for 69 days. [Values are means of three replicates. Numbers in parentheses are standard errors. All means are not significantly different from each other using one-way ANOVA (*F ratio = 1.05* and *p value = 0.431*)].

been previously demonstrated, although metal toxicity varied greatly, depending on soil characteristics and crop type (Kabata-Pendias and Pendias, 1992; Aidid and Okamoto, 1993; Prasad *et al.,* 1999; Davies *et al.*, 2001). In the present study, the data on Cr concentration and accumulation in the Cr (III) and Cr (VI)-treated plants (Tables 4.3 and 4.4) indicate that low amounts of Cr are translocated to the shoots, which may be the reason for no significant retardation of height of *B. juncea*.

At any time of metal exposure and in both Cr oxidation states, the roots of *B. juncea* were affected to a greater degree than the plant shoots (Figure 4.6). For example, at 17 days after sowing, root growth in the Cr (III)-treated moist and air-dry and Cr (VI)-amended moist and air-dry plants was reduced by 36% and 33%, 57% and 54%, respectively, while, in the same Cr and soil treatments, the shoot growth was decreased by 0% and 1%, 18%, and 17%, respectively, as compared to the control plants. The plant roots were found to contain much higher concentrations than the shoots (Table 4.3), which could lead to a remarkable decrease in their dry weight (Figure 4.6).

Root growth was significantly depressed by both Cr species (Figure 4.6). The inhibitory effects of Cr on root growth may have resulted from binding of Cr (III) to the plant tissues and disturbance of osmotic relationships, which lead to the restricted transport of Ca (II) ions across the plasma membrane into the

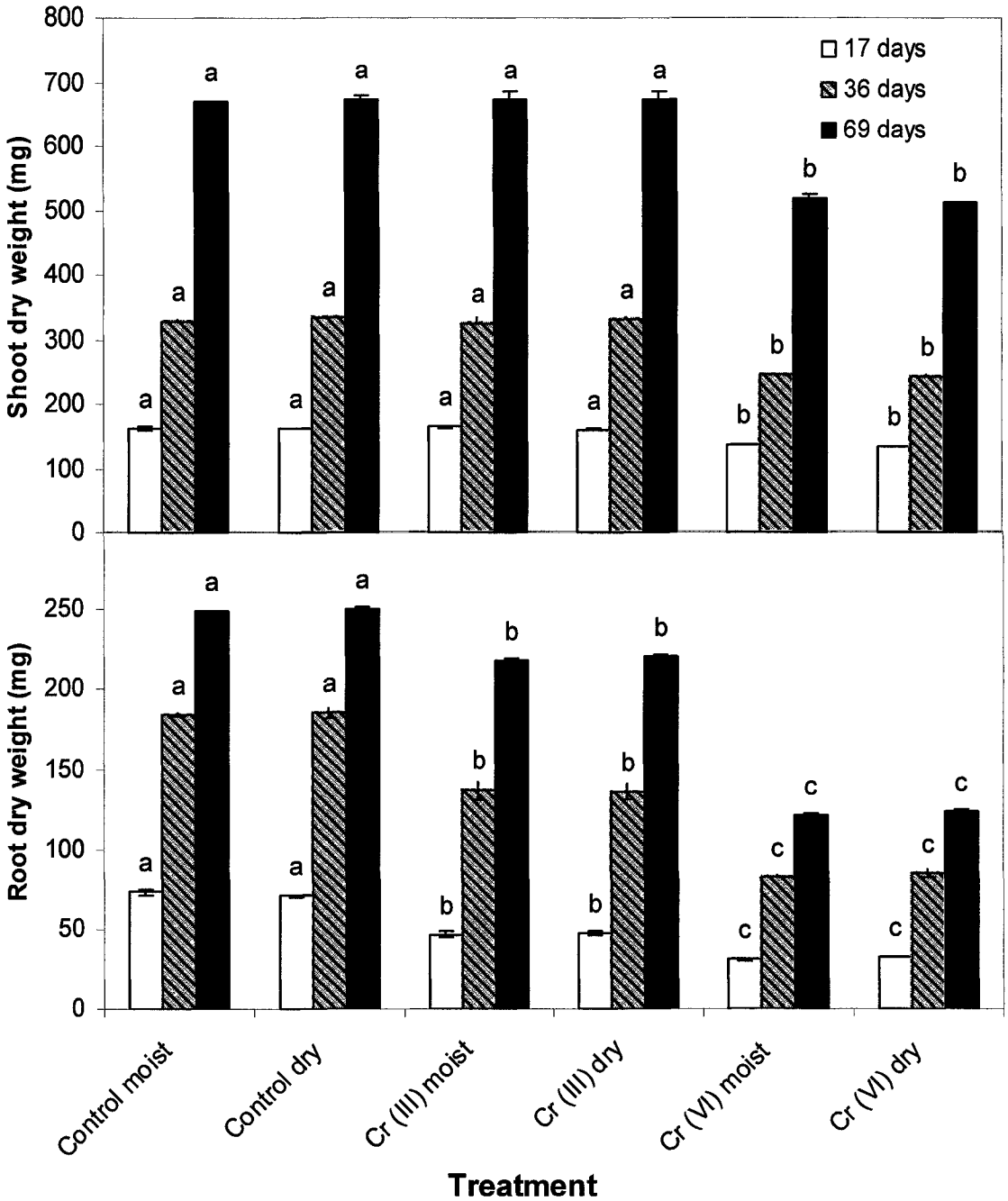

Figure 4.6. Effect of two chromium species [Cr (III) and Cr (VI)] on mean root and shoot dry weight of *Brassica juncea* grown for different exposure periods. [Within each harvest time, means followed by a common letter are not significantly different from each other using one-way ANOVA and LSD test (α = 0.05, n = 3)].

cytoplasm (Liu *et al.*, 1992). In this way, the level of free Ca (II) ions in the cell becomes very low, which leads to a failure of calmodulin (CaM) in activation of a number of key enzymes, including Ca-ATPase (Liu *et al.*, 1992). However, at any exposure period, Cr (VI) appeared to be more toxic than Cr (III). In particular, at 36 days after planting, for Cr (III)-treated plants grown in the moist and air-dry soils, root dry weights were 136 and 135 mg, respectively, whereas, these values dropped to 83 and 85 mg for Cr (VI)-treated plants (Figure 4.6). Since the Cr (III)-treated plants absorbed a similar amount of Cr as those grown in the Cr (VI) treatment (Table 4.4), root growth was expected to be similarly affected by the application of both Cr treatments. However, being a strongly oxidizing agent, Cr (VI) has been linked to structural and ultrastructural alterations in plants (Vazquez *et al.*, 1987). In contrast, Cr (III) is less phytotoxic due to its lower oxidizing potential (Gauglhofer, 1984).

Over the course of the experiment, the dry weight of the plant shoots was significantly inhibited by Cr (VI) treatment, while it was not sensitive to Cr (III) treatment (Figure 4.6). Both higher concentration and accumulation (Tables 4.3 and 4.4), and toxicity of Cr (VI) are likely responsible for this trend.

At any sampling time, there was no significant effect of soil treatment on root and shoot dry weights (Figure 4.6). This was not expected, particularly at the first harvest, when oxidation of a small portion of Cr (III) was observed in the

field-moist soil (Figure 4.1). Nevertheless, the concentration of formed Cr (VI) perhaps was not high enough to cause a significant decrease in the dry weight of roots and shoots.

4.4.4. Chromium influence on plant root exudation

Capillary electrophoresis (CE) was applied to separate, identify, and measure low-molecular-weight organic acids in root exudates of *B. juncea*. This new technique has been proven to allow for a rapid and efficient separation of charged compounds present in small sample volumes (Barbas *et al.*, 1998). Moreover, the separation can often be achieved directly in aqueous media, without sample pretreatment (Barbas *et al.*, 1998). Electrokinetic sample injection is known to enhance CE sensitivity (Galli *et al.*, 2003), which was crucial for the low concentrations of organic acids in this study. The electropherograms of standard mixture of organic acids and solutions of root exudates, collected from the control and Cr (VI)-treated plants at three sampling times, are illustrated in Figures 4.7 and 4.8. It can be seen that malic, citric, succinic, and acetic acids are consistently present in all samples at any harvest time. In addition, at any time of Cr exposure, all peaks in the Cr (VI)-amended plants are higher than those of the control plants (Figures 4.7 and 4.8). Moreover, the magnitudes of absorbance seem to decrease from vegetative (17

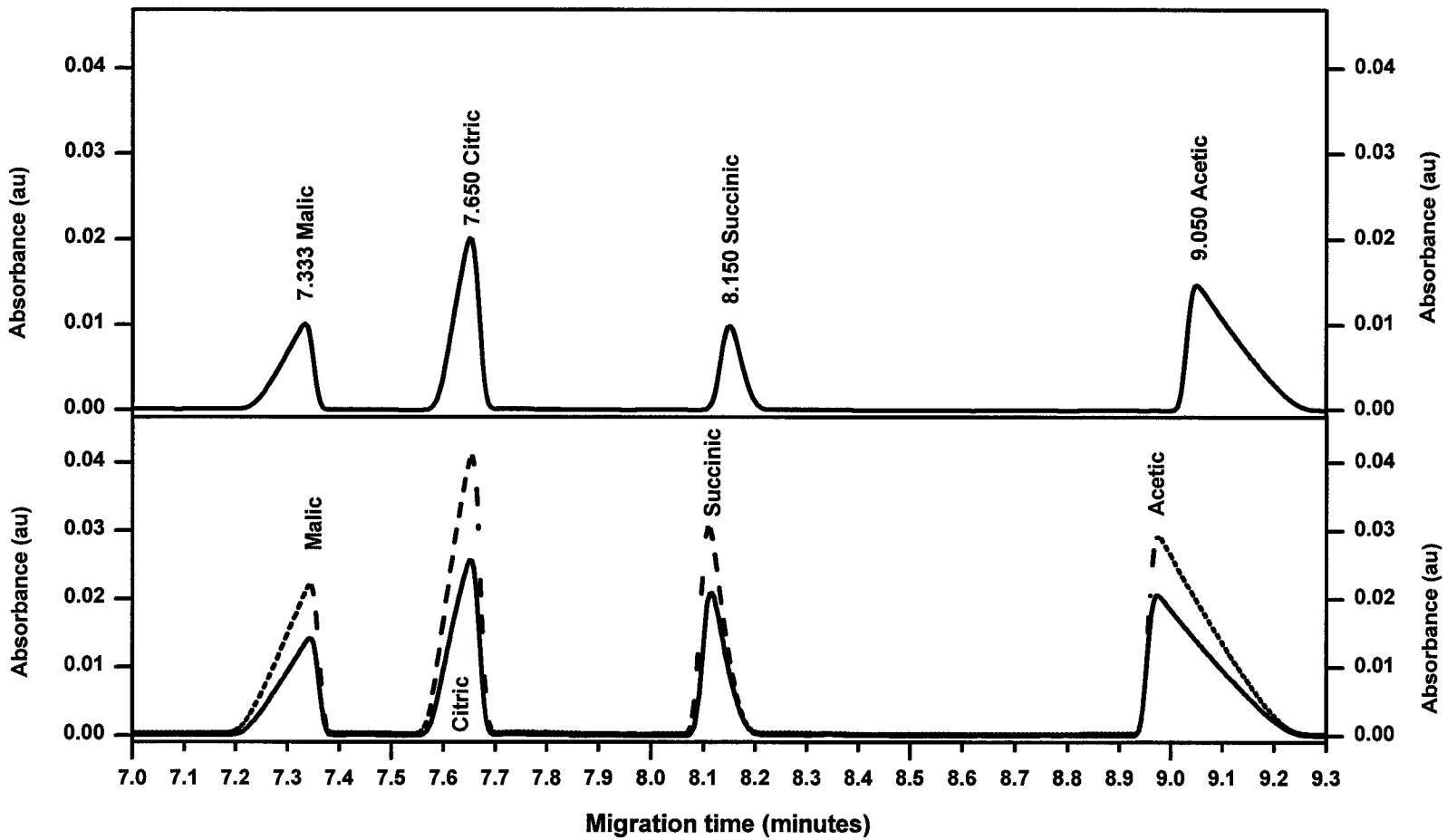

Figure 4.7. Electropherograms of: (a) 10 µM standard solution mixture of malic, citric, succinic and acetic acids; (b) root exudates collected from *Brassica juncea* at 17 days (electrokinetic injection of 10 kV for 10 sec). Solid and dashed graphs represent control and Cr (VI)-treated plants, respectively.

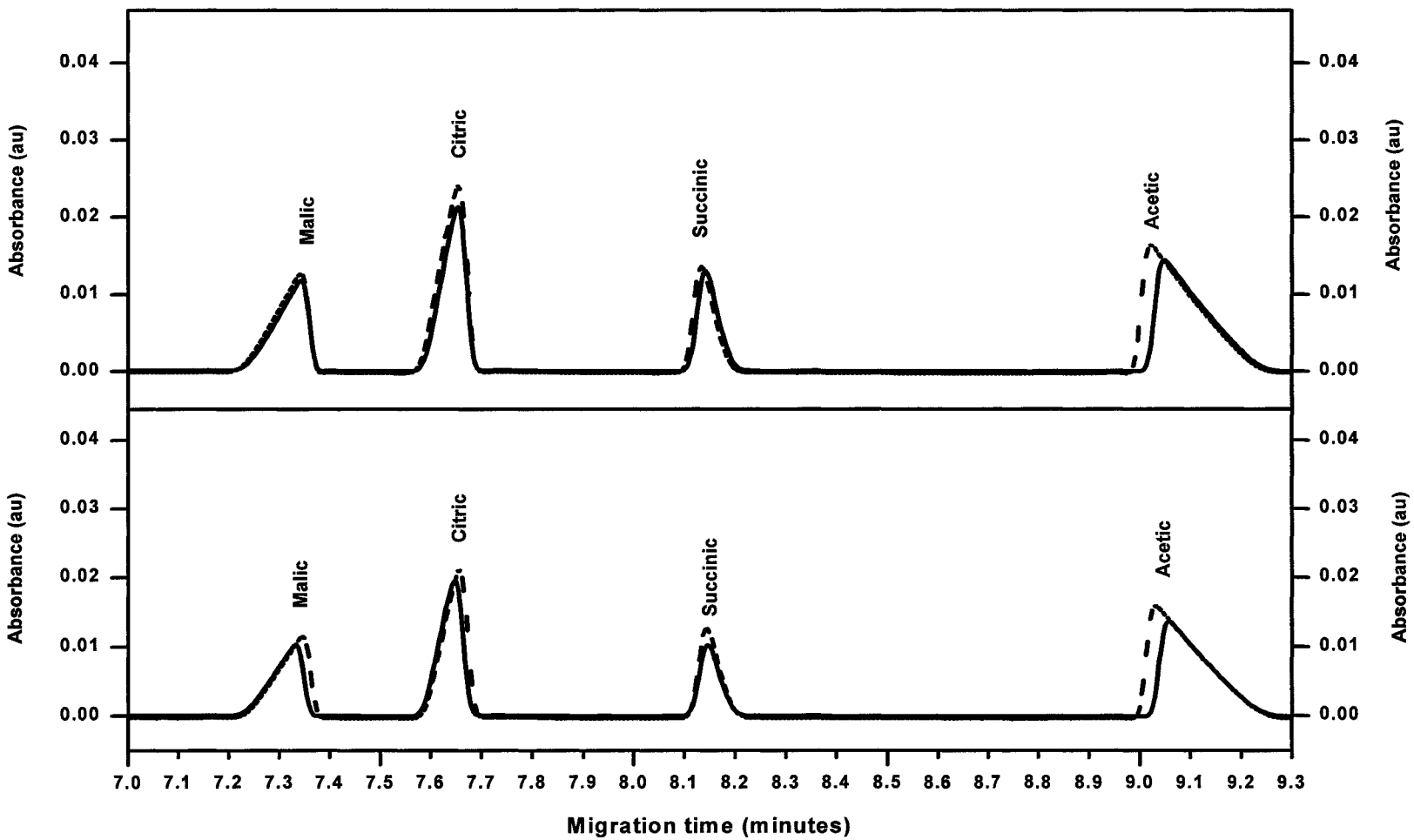

Figure 4.8. Electropherograms of root exudates collected from *Brassica juncea* at: (a) 36 days; (b) 69 days (electrokinetic injection of 10 kV for 10 sec). Solid and dashed graphs represent control and Cr (VI)-treated plants, respectively.

days) to flowering (36 days) and further to fruiting (69 days) stages of plant development (Figures 4.7 and 4.8).

Quantitative data, however, were not collected with the electrokinetic sample injection. Despite the fact that some researchers have determined trace amounts of anions, including organic acids, in different samples (Ehman *et al.*, 1997; Dahlen *et al.*, 2000; Desauziers *et al.*, 2000; O'Flaherty *et al.*, 2001), this type of sample introduction often suffers from matrix bias and poor precision, which was also observed in the present experiment (data not shown), and it is, therefore, not recommended for quantification (Galli *et al.*, 2003). Accordingly, a hydrodynamic or pressure injection, which does not discriminate between the ions, thereby injecting the same effective sample volume of each ion (Devevre *et al.*, 1994), was used for quantification of low-molecular-weight organic acids in the present study. The reproducibility of the quantitative sample introduction using this method is shown in Table 4.6. When an intermediate standard (100 µM) was run six times per day, the standard deviation of migration time varied from 1.2% for citric and acetic acids to 1.3% for malic and succinic acids, respectively, while the standard deviation values for peak areas were 2% for citric and succinic acids and 3% for malic and acetic acids, respectively (Table 4.6).

Table 4.6. Reproducibility of pressure sample introduction of 100 µmol L^{-1} standard mixture of malic, citric, succinic, and acetic acids in capillary electrophoresis.

Organic acid	***Malic***	***Citric***	***Succinic***	***Acetic***
Migration time (min)				
Mean	7.286	7.514	8.009	8.976
STDEV (%)	1.3	1.2	1.2	1.3
Peak area				
Mean	6032	7891	6513	5017
STDEV (%)	3	2	2	3

However, the pressure sample injection appeared to be much less sensitive than the electrokinetic injection (data not shown). Therefore, the samples were concentrated by freeze-drying, just prior to the runs. The same trends as in Figures 4.7 and 4.8 appeared in all samples at all harvest times for three organic acids, namely malic, citric and succinic acids (Figures 4.9 and 4.10). In contrast, acetic acid, detected with the electrokinetic injection (Figures 4.7 and 4.8), seemed to nearly disappear in all samples (Figures 4.9 and 4.10). Being the most volatile, it had probably been lost during the freeze-drying procedure. Other sample preparation techniques, for example, use of anion exchange resins (Robinson, 1986), should therefore be considered for efficient extraction of volatile organic acids.

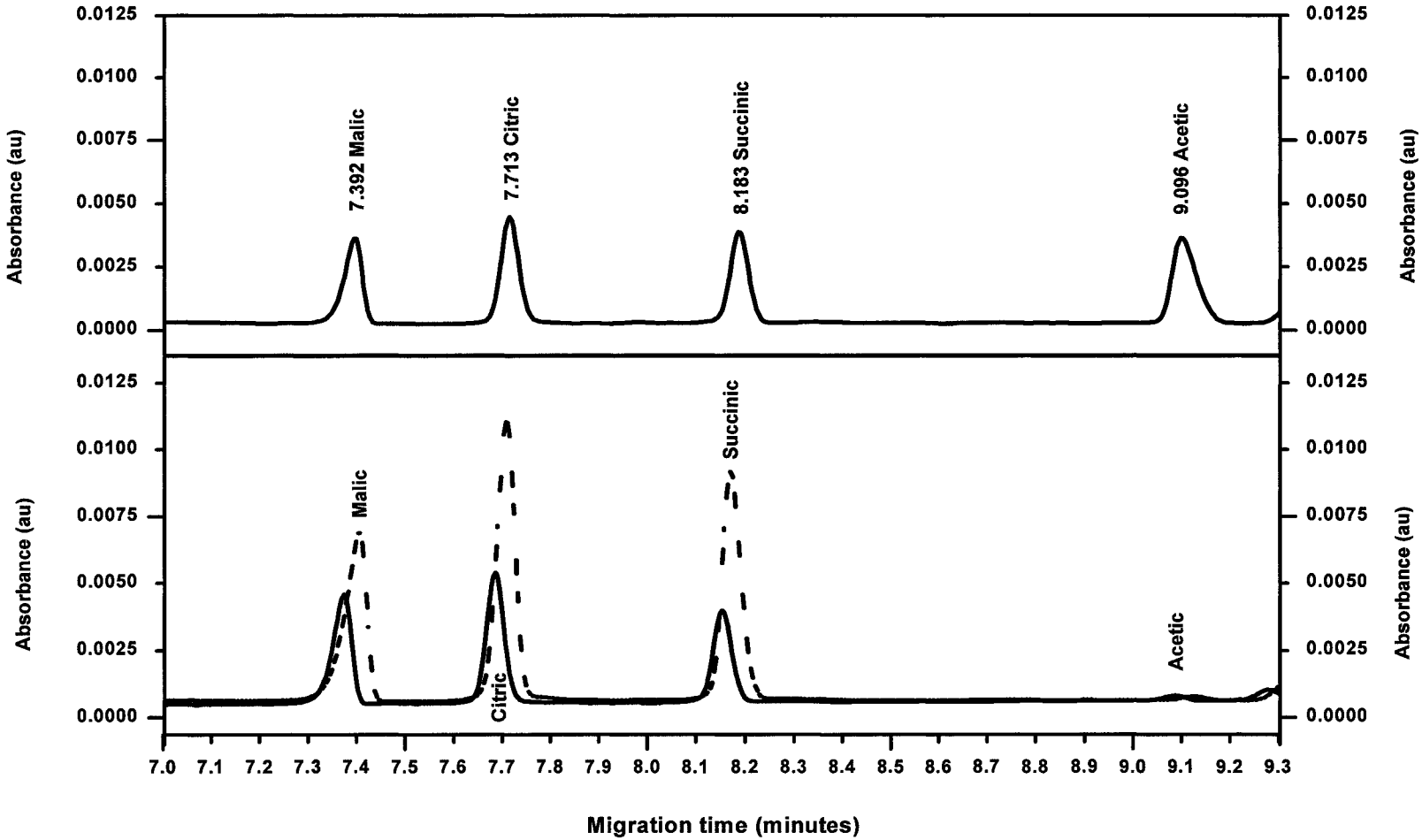

Figure 4.9. Electropherograms of: (a) 100 µM standard solution mixture of malic, citric, succinic and acetic acids; (b) root exudates collected from *Brassica juncea* at 17 days (pressure injection of 0.1 psi for 10 sec). Solid and dashed graphs represent control and Cr (VI)-treated plants, respectively.

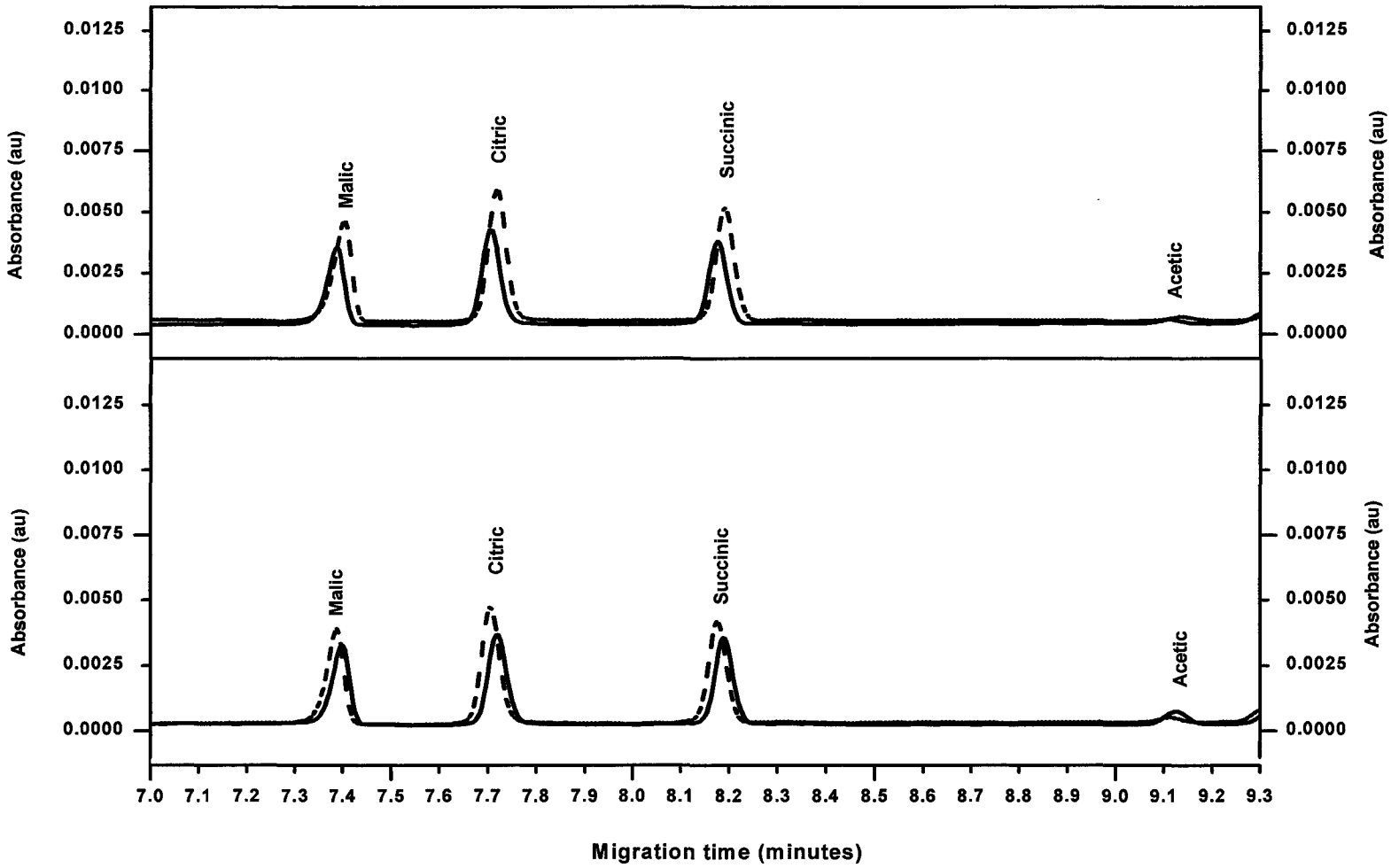

Figure 4.10. Electropherograms of root exudates collected from *Brassica juncea* at: (a) 36 days; (b) 69 days (pressure injection of 0.1 psi for 10 sec). Solid and dashed graphs represent control and Cr (VI)-treated plants, respectively.

In general, root exudation in the rhizosphere occurs as a result of mechanical injury from small insects and nematodes, growth of lateral roots, and abrasive action of soil particles (Rovira, 1969). Low-molecular-weight organic acids released into soils can also increase the ability of plants to survive and grow normally under conditions of nutrient deficiency (Neumann and Romheld, 1999). In particular, increase in organic acid efflux has been observed under K (Kraffczyk *et al.*, 1984), Fe (Mulette *et al.*, 1974), and a general nutrient deficiency (Jones and Darrah, 1995). The organic acid anions in root exudates can chelate Fe and Mn or lower rhizosphere pH, thus making Mn, Fe, and Zn more available for plant uptake (Marschner, 1986). Similarly, organic acid anions can form complexes with Ca and Al present in soil as insoluble phosphates and liberate P uptake by roots (Marschner, 1986). In the present investigation, at any sampling time or with any Cr and soil treatments, citric acid was the most abundant of the three organic acids exuded by *B. juncea* (Table 4.7). The presence of citric acid in root exudates of other plant species is considered to be mostly related to P absorption (Neumann and Romheld, 1999). For example, in *B. napus*, a large increase in succinic, malic, and citric acid levels (by 70 and 12 times, respectively, for malic and citric acids) has been observed under P deficiency (Zhang *et al.*, 1997). However, in the present study, soils were supplied with the N-P-K fertilizer. Therefore, the abundant citrate

Table 4.7. Organic acids (μg L^{-1}) in root exudates of *Brassica juncea* treated with 100 mg kg^{-1} of either $CrCl_3 \cdot 6H_2O$ or $K_2Cr_2O_7$.

Organic acid	**Malic**				**Citric**				**Succinic**			
Treatment	*17 days*	*36 days*	*69 days*	*F ratio and (p value) time*	*17 days*	*36 days*	*69 days*	*F ratio and (p value) time*	*17 days*	*36 days*	*69 days*	*F ratio and (p value) time*
Control moist	452^{a} (3)[1]	284^{c} (5)	131^{e} (1)	*1122.53 (0.000)*	714^{a} (8)	527^{c} (21)	224^{e} (5)	*225.67 (0.000)*	474^{a} (4)	315^{c} (5)	144^{d} (6)	*719.67 (0.000)*
Control dry	453^{a} (3)	285^{c} (8)	132^{e} (5)	*520.44 (0.000)*	713^{a} (21)	543^{c} (15)	230^{e} (6)	*370.95 (0.000)*	476^{a} (8)	309^{c} (7)	152^{d} (4)	*416.06 (0.000)*
Cr (III) moist	455^{a} (4)	293^{c} (11)	129^{e} (2)	*389.22 (0.000)*	718^{a} (13)	539^{c} (6)	230^{e} (5)	*1271.63 (0.000)*	473^{a} (7)	316^{c} (2)	146^{d} (7)	*480.16 (0.000)*
Cr (III) dry	449^{a} (2)	298^{c} (8)	132^{e} (2)	*702.56 (0.000)*	711^{a} (10)	540^{c} (7)	225^{e} (3)	*758.08 (0.000)*	471^{a} (7)	315^{c} (3)	145^{d} (8)	*447.44 (0.000)*
Cr (VI) moist	553^{b} (10)	442^{d} (4)	214^{f} (4)	*471.44 (0.000)*	794^{b} (4)	630^{d} (19)	339^{f} (11)	*215.46 (0.000)*	572^{b} (6)	479^{a} (7)	268^{e} (8)	*327.62 (0.000)*
Cr (VI) dry	549^{b} (8)	450^{d} (13)	217^{f} (6)	*215.19 (0.000)*	793^{b} (7)	635^{d} (18)	342^{f} (10)	*219.10 (0.000)*	571^{b} (6)	480^{a} (5)	272^{e} (6)	*497.60 (0.000)*
F ratio and (p value)												
treatment	*62.10 (0.000)*[2]	*55.39 (0.000)*	*21.17 (0.000)*	-	*7.03 (0.003)*	*71.84 (0.000)*	*38.12 (0.000)*	-	*39.56 (0.000)*	*175.07 (0.000)*	*64.71 (0.000)*	-

[1] Standard error. [2] p value.

Within each acid, means followed by a common letter are not significantly different from each other using one-way ANOVA and LSD test ($\alpha = 0.05$, $n = 3$).

production could not be related to the low P concentration in the studied soils. On the other hand, the amount of other nutrients, Fe, for example, which appears to be limited in the soils under present investigation (Fe_p = 0.68%: Arocena and Sanborn, 1999), might have been insufficient for normal growth of *B. juncea*, thereby enhancing citric acid exudation. There are reports in the literature concerning changes in concentrations of organic acids in the roots of several plant species induced by Fe deficiency. As an example, citrate and to a lesser extent malate increased with Fe deficiency in roots of *H. annuus* (Venkat Raju *et al.*, 1972). Citric and malic acids are not the only organic acids which exudation is enhanced in Fe-deficient roots. For instance, Alhendawi *et al.* (1997) found that increase in bicarbonate in nutrient solution led to chlorosis and to increase of citrate, malate, aconitate, and succinate in the roots of *H. vulgare*, *Sorghum bicolor* (sorghum), and *Z. mays*.

The solubilizing ability of organic acids has been reported to be parallel to their metal-binding capacity, which, in turn, is correlated with their dissociation constants (Srivastava *et al.*, 1998a). The order of the excretion of the organic acids obtained in the present study (Table 4.7) is in agreement with the dissociation constants, i.e., Ka_1, Ka_2, for citric acid ($7.45x10^{-4}$, $1.73x10^{-5}$), malic acid ($3.48x10^{-4}$, $8.0x10^{-6}$), and succinic acid ($6.21x10^{-5}$, $2.31x10^{-6}$) (Martell and Smith, 1976).

Table 4.7 also demonstrates that, at any collection time, exposure of plants to Cr (VI) caused a significant increase in concentration of each organic acid, while Cr (III) treatment did not affect root exudation of *B. juncea*. Organic acids such as citric, malic, and aspartic acids in root exudates of *Z. mays* (Srivastava *et al.*, 1998a), citric, oxalic, and aspartic acids in *Lycopersicon esculentum* (tomato) (Srivastava *et al.*, 1998b), and oxalic, malic, and glycine in *Triticum vulgare* (brad wheat) (Srivastava *et al.*, 1999), have been reported to have a potential for reducing Cr (VI) and chelating either formed or present Cr (III). As a result, enhanced root exudation, observed in this study with Cr (VI) treatment, could be a defense (detoxification) mechanism of the plant. On the other hand, the concentrations of organic acids (Table 4.7) seem insufficient for the binding of each of the Cr (III) and Cr (VI) present in the roots (Table 4.3). This could be due to utilization of some portion of root exudates by rhizospheric microorganisms, which may have still been adhered to the roots after a gentle wash and sonication. As Cr (III) did not increase exudation of organic acids, while Cr (VI) did, it is also possible that there was simple leakage of organic acids through ruptured plasma membranes, caused by high oxidative potential of Cr (VI).

In all treatments, the highest levels of any acid were detected at the vegetative stage (17 days after planting). These levels decreased towards flowering and fruiting stages (36 and 69 days after planting, respectively) of plant

development (Table 4.7). Seedling development has a high nutrient demand not only for growth of individual organs, synthesis of new cytoplasm and sub-cellular organelles and cell walls, but also for cell division and expansion (Moorby and Besford, 1983). In contrast, during the reproductive stage, growth of plant slows. Furthermore, at maturity, the plant does not produce much chemical energy (photosynthetic rates slow down) and requires very little nutrients (Spaugh, 1999). This is one possible explanation why root exudation declined with time (Table 4.7). These findings are in agreement with a previous study of Lucas Garcia *et al.* (2001), who observed 50 and 80% decrease in the total amount of organic acids exuded by *Lupinus albus* (white lupine) and *Lupinus luteus* (European yellow lupine), respectively, at the fruiting stage compared to that at the flowering stage of plant development. Further, the rate of leakage of oxalic and succinic acids was greater from the youngest seedlings than from older mycorrhizal and non-mycorrhizal plant species (Schwab *et al.*, 1983).

4.5. Microscopic effects of chromium on plant growth

4.5.1. Chromium influence on shoot anatomical characteristics

The stem diameter significantly decreased in Cr (VI) treatment with a reduction in the width of primary xylem and number of both vascular bundles and xylem cells (Table 4.8). A small amount of Cr, either soluble Cr (VI) or

Table 4.8. Effect of two chromium species [Cr (III) and Cr (VI)] on number of vascular bundles and xylem cells, stem diameter, and width of epidermis, cortex, primary (1^0) and secondary (2^0) phloem and xylem, and pith in *Brassica juncea* after 69 days of growth.

Treatment	*Stem diameter (mm)*	*Number*		*Width (%)*						
		vascular bundles	*xylem cells*	*Epidermis*	*Cortex*	*Phloem* 1^0	*Phloem* 2^0	*Xylem* 1^0	*Xylem* 2^0	*Pith*
Control moist	2.6^a (0.0)[1]	37^a (1.2)	14^a (0.0)	1.5^a (0.0)	6.6^a (0.1)	2.8^a (0.2)	1.8^a (0.2)	15^a (0.2)	9.0^a (0.3)	64^a (0.6)
Control dry	2.7^a (0.0)	36^a (0.7)	13^a (0.8)	1.7^a (0.2)	6.2^a (0.4)	2.8^a (0.2)	1.8^a (0.2)	14^a (0.1)	9.0^a (0.4)	64^a (0.2)
Cr (III) moist	2.6^a (0.0)	35^a (0.7)	13^a (0.7)	1.4^a (0.2)	6.9^a (0.5)	2.7^a (0.2)	1.5^a (0.0)	15^a (0.2)	8.5^a (0.3)	64^a (1.0)
Cr (III) dry	2.6^a (0.0)	35^a (0.5)	15^a (0.7)	1.5^a (0.0)	7.2^a (0.5)	2.8^a (0.2)	1.8^a (0.2)	14^a (0.1)	9.2^a (0.3)	63^a (0.5)
Cr (VI) moist	2.2^b (0.0)	19^b (0.5)	11^b (0.3)	1.8^a (0.0)	8.0^a (0.3)	3.4^a (0.2)	2.2^a (0.5)	9.2^b (0.4)	10^a (0.1)	65^a (0.5)
Cr (VI) dry	2.1^b (0.0)	16^b (1.4)	10^b (0.5)	1.9^a (0.0)	7.3^a (0.9)	3.2^a (0.2)	1.9^a (0.0)	9.2^b (0.9)	11^a (0.7)	66^a (0.8)
F ratio and (p value)	*43.29 (0.000)*[2]	*67.57 (0.000)*	*7.05 (0.003)*	*1.87 (0.174)*	*0.73 (0.612)*	*0.37 (0.859)*	*0.30 (0.905)*	*41.42 (0.000)*	*1.53 (0.252)*	*1.75 (0.198)*

[1] Standard error. [2] p value. Within each column, means followed by a common letter are not significantly different from each other using one-way ANOVA and LSD test ($\alpha = 0.05$, n = 3).

reduced Cr (III), can be transported to the upper plant parts by an active mechanism via the transpiration stream in xylem or by a mechanism similar to that of Ca^{2+} (Skeffington *et al.*, 1976; Barcelo *et al.*, 1985). Toxic concentrations of Cr decrease the number and diameter of xylem cells (Barcelo and Poschenrieder, 1990). In a similar study, the sizes of both phloem and xylem cells decreased in stems of Cr (VI)-treated *P. vulgaris* (Vazquez *et al.*, 1987). Suseela *et al.* (2002) also observed a decrease in the number of fibers in the shoot of *S. lacustris*, treated with 8 mg L^{-1} Cr (VI) for 30 days, as an indicator of pollution with Cr (VI). The inhibition of these plant characteristics may be a result of interference of Cr (VI) with cell division and cell elongation that leads to decrease in cell water content (Barcelo and Poschenrieder, 1990). Heavy metals have been shown to affect both processes in plants. This could be due to insufficient supply of nutrients (Barcelo *et al.*, 1985) and plant growth regulators from the affected roots, which may in turn influence the differentiation of tissues in stems (Setia and Bala, 1994). For example, limited supply of cytokinins, which are mainly synthesized in roots (Van Staden and Davey, 1979), has been linked to inhibition of lateral shoot development during Al toxicity stress (Pan *et al.*, 1988).

The leaves were not affected by any Cr and soil treatment (Table 4.9). Some changes in the plant leaves were expected, since some visual stress was

Table 4.9. Effect of two chromium species [Cr (III) and Cr (VI)] on leaf thickness, thickness of palisade and spongy mesophyll, palisade cell layer number, and leaf vascular bundle number of *Brassica juncea* after 69 days of growth.

Treatment	*Leaf thickness (μm)*	*Palisade mesophyll thickness (μm)*	*Spongy mesophyll thickness (μm)*	*Number of palisade cell layers*	*Number of vascular bundles*
Control moist	379 (4)[1]	160 (9)	167 (8)	4 (0.0)	2 (0.0)
Control dry	377 (0.0)	161 (2)	167 (5)	4 (0.2)	1 (0.2)
Cr (III) moist	376 (6)	168 (7)	161 (5)	4 (0.2)	2 (0.5)
Cr (III) dry	361 (10)	166 (7)	168 (3)	4 (0.0)	2 (0.0)
Cr (VI) moist	369 (6)	157 (5)	167 (2)	4 (0.0)	1 (0.5)
Cr (VI) dry	366 (13)	154 (11)	161 (4)	4 (0.0)	2 (0.2)
F ratio and (p value)	*0.43 (0.817)*[2]	*0.26 (0.928)*	*0.23 (0.944)*	*1.20 (0.366)*	*0.88 (0.523)*

[1] Standard error. [2] p value.

Within each column, means are not significantly different from each other using one-way ANOVA ($\alpha = 0.05$, $n = 3$).

observed in the Cr-treated plants (Table 4.5). However, only one leaf, at the 3rd node, was studied. This suggests that Cr-injured or relatively immature leaves should also be collected, since an individual leaf may not be representative of leaves of the entire plant.

4.5.2. Chromium influence on root anatomical characteristics

The results indicate that tap and lateral roots of *B. juncea* were sensitive to Cr, although soil treatment did not significantly affect any anatomical characteristics of the roots (Table 4.10). Roots are generally the first organs to contact toxic metals in soils and they usually accumulate significantly higher amounts than the aerial plant parts (stems and leaves) (Breckle, 1989). In the present study, most of the Cr was taken up and retained by the plant roots (Tables 4.3 and 4.4), which may also explain why the cells of the plant leaves did not show any metal injury (Table 4.9). In past reports on Cr in plants, it has been hypothesized that the reduction of toxic Cr (VI) to less toxic Cr (III) occurs in the roots (Parr and Taylor, 1980; Micera and Dessi, 1988; Lytle *et al.*, 1998; Aldrich *et al.*, 2003). However, when the Cr (VI) concentration within cells exceeds the reducing capacity of cells, toxicity might occur (Vazquez *et al.*, 1987; Kortenkamp *et al.*, 1991). On the other hand, the amount of Cr (VI) formed in the Cr (III)-treated field-moist bulk soil (Figure 4.1) was probably insufficient to

Table 4.10. Effect of two chromium species [Cr (III) and Cr (VI)] on root diameter, xylem diameter, number and width of all cells in a part of xylem and large cells in entire xylem of tap and lateral roots of *Brassica juncea* after 69 days of growth.

Treatment	*Root diameter (μm)*		*Xylem diameter (μm)*		*Number of large cells in entire xylem*		*Width of large cells in entire xylem (μm)*		*Number of cells in part of xylem*		*Width of cells in part of xylem (μm)*	
	Tap root	*Lateral root*	*Tap root*	*Lateral root*	*Tap root*	*Lateral root*	*Tap root*	*Lateral root*	*Tap root*	*Lateral root*	*Tap root*	*Lateral root*
Control moist	1044^{a} (29)[1]	187^{a} (1)	909^{a} (16)	102^{a} (2)	6^{a} (0.5)	4^{a} (0.3)	36^{a} (1)	20^{a} (0.0)	43^{a} (1)	16^{a} (0.3)	17^{a} (0.0)	12^{a} (2)
Control dry	1017^{a} (16)	192^{a} (0.4)	883^{a} (10)	112^{a} (20)	6^{a} (0.3)	3^{a} (0.0)	41^{a} (2)	22^{a} (3)	45^{a} (1)	16^{a} (0.0)	18^{a} (2)	12^{a} (1)
Cr (III) moist	744^{b} (24)	194^{a} (5)	644^{b} (80)	112^{a} (11)	4^{b} (0.0)	2^{a} (0.3)	32^{b} (0.0)	19^{a} (1)	63^{b} (1)	20^{a} (1)	12^{b} (1)	10^{a} (1)
Cr (III) dry	766^{b} (20)	185^{a} (9)	616^{b} (57)	110^{a} (0.0)	4^{b} (1.0)	3^{a} (0.0)	33^{b} (1)	20^{a} (1)	61^{b} (3)	19^{a} (1)	13^{b} (1)	9^{a} (1)
Cr (VI) moist	622^{c} (33)	131^{b} (3)	396^{c} (25)	73^{b} (0.0)	2^{b} (0.5)	2^{a} (0.4)	28^{c} (1)	16^{a} (1)	97^{c} (3)	20^{a} (1)	9^{c} (1)	8^{a} (1)
Cr (VI) dry	706^{c} (14)	132^{b} (8)	465^{c} (16)	73^{b} (1)	2^{b} (0.0)	2^{a} (0.5)	29^{c} (1)	17^{a} (1)	92^{c} (7)	20^{a} (1)	9^{c} (1)	7^{a} (1)
F ratio and (p value)	*35.34 (0.000)*[2]	*15.63 (0.000)*	*16.32 (0.000)*	*3.43 (0.046)*	*8.39 (0.001)*	*1.75 (0.211)*	*19.00 (0.000)*	*1.53 (0.265)*	*28.22 (0.000)*	*2.80 (0.078)*	*9.08 (0.001)*	*1.49 (0.278)*

[1] Standard error. [2] p value. Within each column, means followed by a common letter are not significantly different from each other using one-way ANOVA and LSD test ($\alpha = 0.05$, $n = 3$).

significantly inhibit any anatomical characteristics of either tap or lateral roots (Table 4.10).

The tap or primary roots seemed to exhibit changes in both Cr (III) and Cr (VI) treatments with the latter being more toxic to the plants than the former (Table 4.10, Figure 4.11). In particular, the root and xylem diameters were considerably reduced in the plants grown in the Cr (VI) treatment compared to those in the Cr (III) treatment; the control plants had significantly lower values compared to those treated with Cr (III). Moreover, the number of large cells in the entire xylem was significantly higher for the control plants compared to the Cr (III, VI)-treated plants, while the width of these cells was significantly lower for the Cr (VI) plants compared to both control plants and those grown in Cr (III) treatment. The similar trend was observed for the width of cells in a part of xylem; however, the number of these cells was significantly higher in the Cr (VI)-treated plants compared with either the control or Cr (III)-treated plants (Table 4.10). The higher toxicity effect of Cr (VI) on plant roots was expected. It is known that *B. juncea* can absorb both Cr (III) and Cr (VI); however, the latter is taken up more easily and in higher concentrations than the former (Kumar *et al.*, 1995; Shahandeh and Hossner, 2000). In the present study, the root growth was severely inhibited in the Cr (VI)-treated plants (Figure 4.6), thus possibly lowering the capacity of the plant to take up water from the soil. In general, the primary toxic effects of heavy metals are their influence on membrane function,

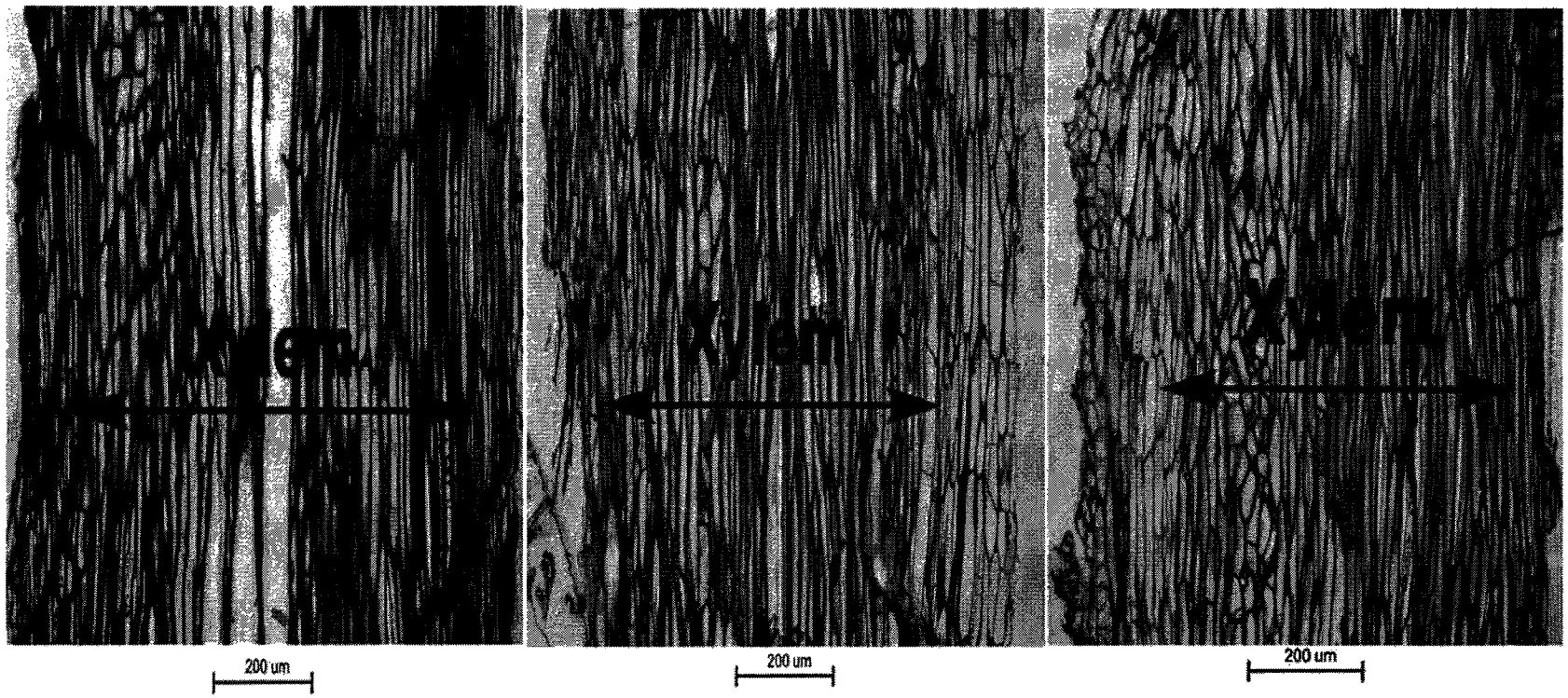

Figure 4.11. Effect of Cr on tap root anatomy. Longitudinal sections (100x) of tap roots from: (a) control, (b) Cr (III)-treated, and (c) Cr (VI)-treated *Brassica juncea* after 69 days of growth in field-moist soils. Note the smaller root and xylem diameters and a number of large xylem cells in (b) and further reduction of these root characteristics in (c) in comparison to (a). Cr (VI) treatment (c) also caused the greatest increase in the number of cells in the part of xylem (chosen for measurements) compared to either the control (a) or Cr (III) treatment (b).

ion balance and enzyme activity, which could bring substantial alterations of water relations at both the cellular and the whole plant level (Barcelo and Poschenrieder, 1990). Cell wall extensibility and thus cell expansion have been reported to be severely inhibited due to metal-induced decrease of cell wall synthesis (Barcelo and Poschenrieder, 1990). For example, Cr (VI) has been found to alter Golgi activity (Vazquez *et al.*, 1987). Being a strongly oxidizing agent, Cr (VI) appears to damage various cellular components including cell wall and membranes resulting in their structural change (Vazquez *et al.*, 1987). This may in turn reduce membrane water permeability and, as a result, contribute to reduced water uptake. Decrease of vessel diameter in plants is also one of the effects caused by Cr toxicity (Barcelo and Poschenrieder, 1990). There are several reports on the influence of Cr on the vascular system of roots. For example, the vessel density, the dimension of vessel elements and number of fibers all have decreased significantly in the root of *S. lacustris* treated with Cr (VI) (Suseela *et al.,* 2002). In the present study, the narrower xylem elements in the Cr (III, VI)-treated plants compared to elements in the control plants may have lead to decrease in root and xylem diameters. It is generally suggested that the growth of the stelar or vascular tissue of the roots is regulated by the activity of meristematic tissue and plant growth regulators (Burstrom and Svensson, 1972). Cytokinins and auxins are known to promote xylem differentiation (Dalessandro and Roberts, 1971; Dalessandro, 1973; Minocha and Halperin, 1974; Aloni, 1987). Aloni (1987) proposed a hypothesis according to which the

rate of conduit, i.e., production of xylem vessels, is positively correlated with the amount of auxins that the differentiating cells receive. Moreover, the final size of a conduit is determined by the rate of cell differentiation. Cell expansion ceases after the secondary wall is deposited; therefore, rapid differentiation results in narrow vascular elements, while slow differentiation permits more cell expansion and therefore results in wide vascular elements. Conduit density is also controlled and positively correlated with auxin concentration (Pizzolato, 1982; Aloni and Zimmermann, 1983). Auxins probably do not act alone, but interact with cytokinins, which generally stimulate cell division (Boote, 1977). The results in this study suggest that Cr (VI) decreased the size, but increased the density of xylem cells perhaps through high concentrations of auxins and cytokinins. In addition, the high density of the very narrow xylem cells in the Cr (III, VI)-treated plants in this study may be a defensive response of *B. juncea* to metal stress. However, there is a lack of experimental data on this topic and further studies are required to investigate this phenomenon.

In the lateral roots, the values for root and xylem diameters were significantly lower for the Cr (VI)-treated plants, while remained similar for those exposed to Cr (III) compared to control (Table 4.10 and Figure 4.12). However, the number and the width of the large cells in the entire xylem, the number of cells in a part of xylem, and the width of these cells were not significantly

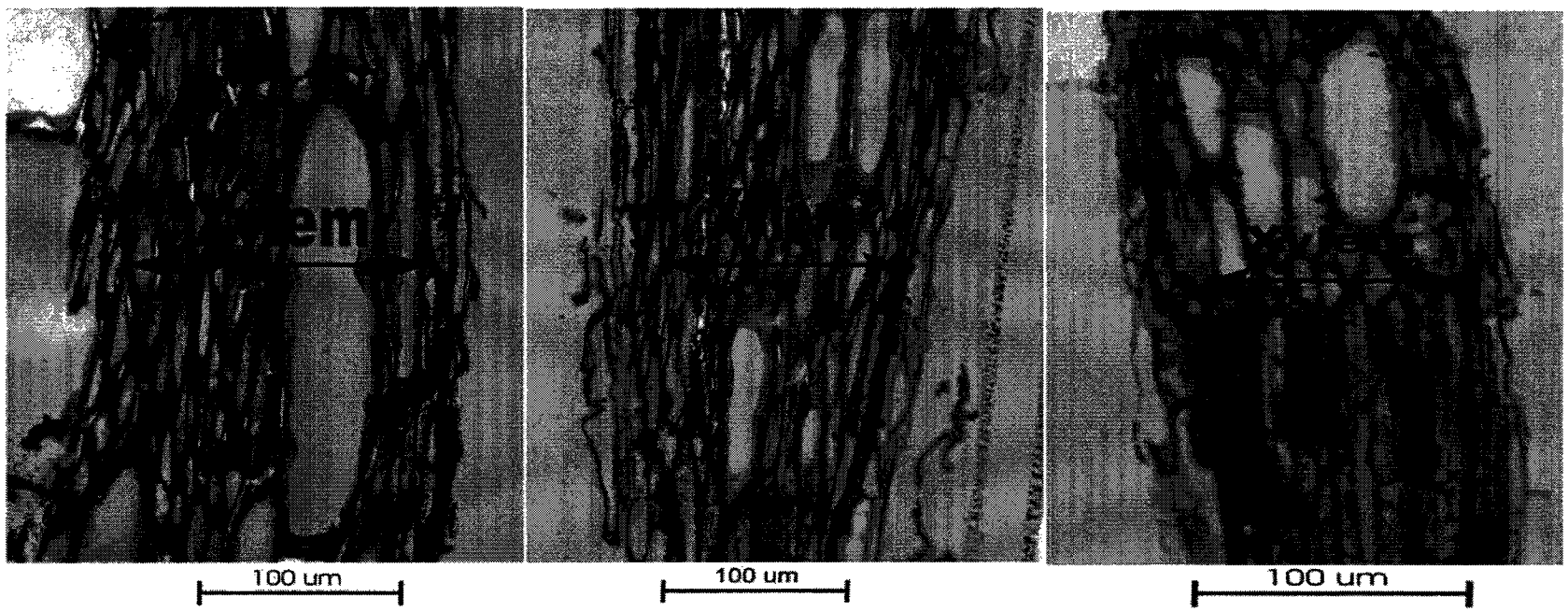

Figure 4.12. Effect of Cr on lateral root anatomy. Longitudinal sections (200x) of lateral roots from: (a) control, (b) Cr (III)-treated, and (c) Cr (VI)-treated *Brassica juncea* after 69 days of growth in field-moist soils. Note the decrease in root and xylem diameters in Cr (VI) treatment (c) in comparison to control (a) and Cr (III) treatment (b) and the absence of any significant effect of both Cr treatments on the number and width of large xylem cells in the entire xylem and in the part of xylem chosen for measurements.

affected by any Cr treatment (Table 4.10). A major function of lateral roots is believed to be nutrient and water uptake as well as mycorrhizal formation, while a major function of tap roots is primarily mechanical support and transport of water to shoots (Raven *et al.*, 1999). Therefore, higher Cr toxicity would be expected in the lateral rather than in the tap roots. However, several reports in the literature have demonstrated the extremely high resistance of lateral roots to heavy metals (Ivanov, 1994; Seregin and Ivanov, 1998). Researchers believe that this phenomenon is due to the barrier properties of the endodermis in lateral roots compared with that in tap roots. In particular, while the transport of heavy metals across the endodermal barrier, i.e., the Casparian strip, is hampered, Moon (1986) found that laterals can interrupt the continuity of the endodermis for a brief time, thereby forming gaps for metal transport (Wierzbicka, 1987; Ksiazek and Wozny, 1990; Seregin and Ivanov, 1997). Therefore, due to more limiting transport of Cr to the root vascular system and further to shoots, in this experiment, the tap root cells might have had higher Cr concentrations than the lateral root cells. As a result, the former would be more seriously damaged than the latter, which was indeed the case (Table 4.10).

4.5.3. Chromium speciation and distribution within plant

Figure 4.13 shows the XANES data and LC-XANES fittings for the Cr (VI)-treated root and leaf of *B. juncea* grown in field-moist soil for 69 days. The lack of a pronounced pre-edge peak of Cr (VI), present in the

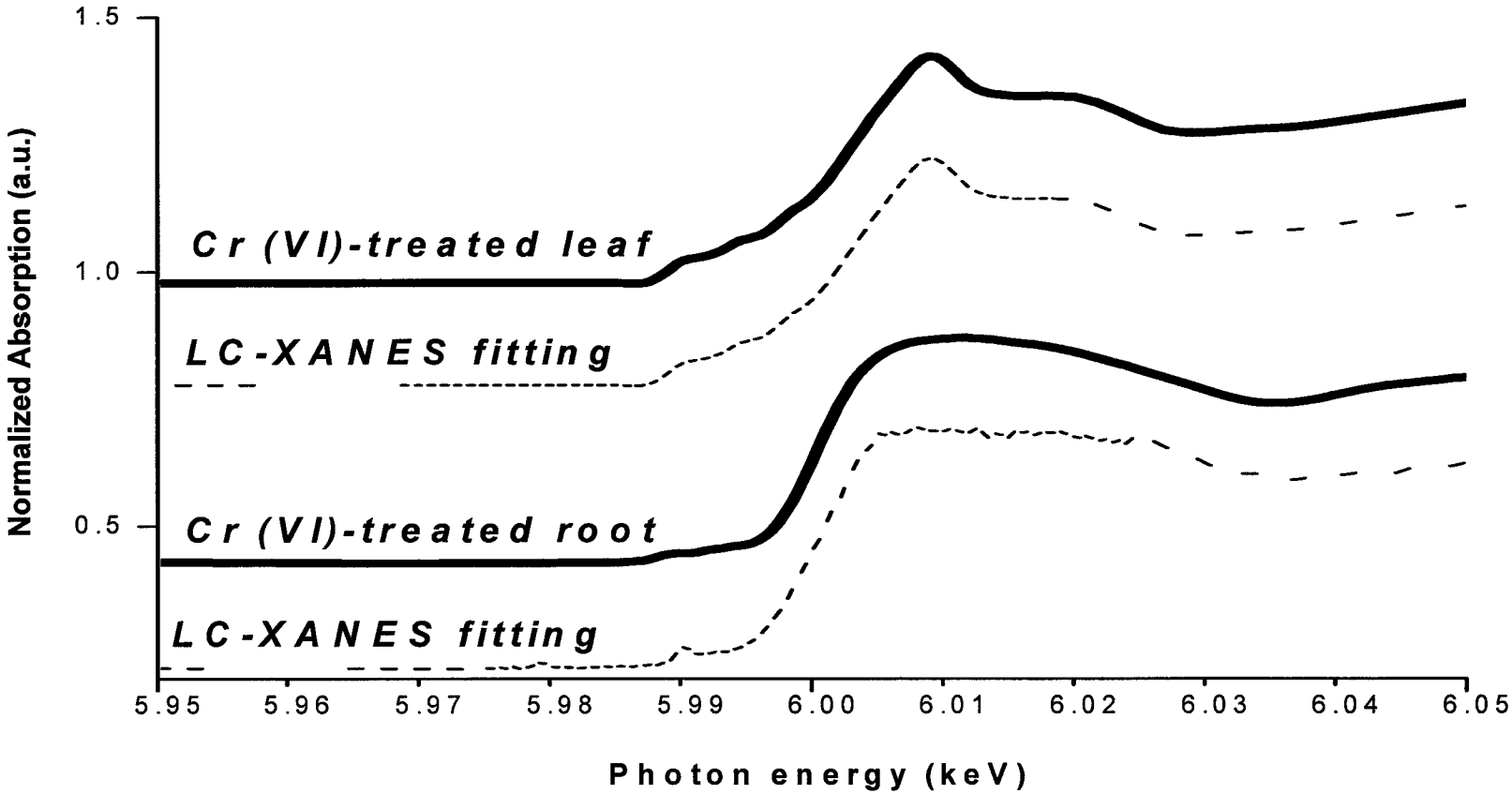

Figure 4.13. XANES spectra and LC-XANES fittings of Cr (VI)-treated leaf and root of *Brassica juncea* grown for 69 days in the field-moist soil.

reference compound of Cr (VI)-dichromate (Figure 4.2), indicates that, in both the root and leaf, the Cr exists as Cr (III). Therefore, *B. juncea* converted the more toxic Cr (VI) into the less toxic Cr (III). The conversion clearly occurred in root tissues, since most of Cr (III) was observed in the plant roots (Figure 4.13). In studies that used similar XAS technique, both the lateral roots and leaves of *E. crassipes*, supplied with Cr (VI) in nutrient solution, contained only Cr (III) (Lytle *et al.*, 1998). In addition, Zayed *et al.* (1998) reported the absence of Cr (VI) species in the roots of 4- to 5-week-old *B. oleracea* and *Brassica rapa* (turnip). Yet in another study, the XAS results demonstrated that Cr (VI), taken up by the roots of *Prosopis* sp. (mesquites), was fully reduced to Cr (III) and transported to the leaf of the plant (Aldrich *et al.*, 2003).

Skeffington *et al.* (1976) studied Cr uptake and transport in *H. vulgare* seedlings. The authors hypothesized that Cr is transported largely via xylem. The more mobile Cr (VI) apparently moves more easily than the less mobile Cr (III) due to retention of the latter by ion exchange on vessel walls (Skeffington *et al.*, 1976), as it happens for Ca (II) (Shewry and Peterson, 1974). Cr (III)-organic acid complexes are water-soluble and, thus, are mobile. They are not retarded by ion exchange and, therefore, move more quickly than Cr (III) in roots and shoots. The mechanism(s) by which plants can reduce Cr (VI) to Cr (III) may be related to the formation of such complexes. For example, Aldrich *et al.* (2003) found high amounts of Cr (III) acetate (about 58%) in the roots of *Prosopis* spp.

Due to root respiration, the amount of CO_2 at the root surface is generally high (Paul and Clark, 1989). The authors therefore hypothesized that Cr (VI) was possibly reduced via an oxidation-reduction reaction with an acetate-type ligand, given the high negative potential of the redox pair of CO_2/acetate (Aldrich *et al.*, 2003). In another study (Lytle *et al.*, 1998), it was also found that Cr was bound to oxalate, a low-molecular-weight ligand. *B. juncea* might have the similar mechanism of Cr (VI) reduction via ligand formation with a low-molecular-weight organic acid. The XANES data on the fitting of Cr (III, VI) model compounds are illustrated in Table 4.11. In the root samples of the Cr (VI)-treated plants, from the reference compounds used in the present study, the best fits were found with Cr (III) as Cr (III)-acetate present at 72% as well as with a mixture of Cr (III)-oxalate (14%) and Cr (III)-formate (9%). A small amount of Cr (VI) as $K_2Cr_2O_7$ (5%) was also present in the roots (Table 4.11), thereby confirming that nearly complete conversion of Cr (VI) to Cr (III) occurred. One possibility of how Cr (III) enters the roots is that secreted acetic acid, which was qualitatively identified in root exudates of *B. juncea* (Figures 4.7 and 4.8), could have reduced Cr (VI) externally and then chelated Cr (III) may have been taken up by the roots, while some Cr (VI) could have been transported directly into the root cells. However, quantitative data on acetic acid is needed to confirm this. Additionally, the presence of low concentrations of other organic acids detected in the root exudates (Table 4.7) suggests that their accumulation could not confirm the observed Cr (VI) reduction. In this study, roots were not analyzed for internal

organic acids that are found to be quite different in quality and quantity from the root exudates (Gleba *et al.*, 1999). Therefore, the concentrations of the internal organic acids might have been sufficient enough to promote the Cr (VI) reduction.

Table 4.11. Distribution of chromium compounds (%) in a root and leaf of *Brassica juncea* grown for 69 days in field-moist soil treated with 100 mg kg^{-1} of $K_2Cr_2O_7$ (n = 2).

Reference compound	*Root*	*Leaf*
Cr (VI)-dichromate	5	0
Cr (III)-chloride hexahydtared	0	0
Cr (III)-formate	9	0
Cr (III)-acetate	72	19
Cr (III)-trioxalate	14	81

In the leaf tissues, Cr (III)-oxalate (81%) and Cr (III)-acetate (19%) species were detected (Table 4.11). These findings suggest that Cr (III), chelated by acetate externally in the plant rhizosphere, could have been transported to the leaf cells to form a complex with internal oxalic acid in the vacuoles, which are well-known storage compartments of large amounts of organic acids in plants (Hodgkinson, 1977). On the other hand, if Cr (III) was chelated by acetate internally, it could have moved from the cytosol of the root cells, which occupies a small proportion of the cell volume (Rauser, 1999), into the major part of root cells, i.e., the vacuole, to produce Cr (III)-oxalate and release acetate for return to the cytosol. Nevertheless, future studies on internal

concentrations of organic acids, including oxalic and acetic acids, in root and leaf tissues as well as on distribution of these organic acids between the cytosol and the vacuoles are required to confirm these speculations.

The distribution of Cr (white color) in a cross-section of a leaf and a longitudinal section of a lateral root is presented in Figures 4.14 and 4.15. The X-ray image of the Cr (III)-treated leaf shows enriched Cr accumulation in the lower spongy mesophyll and the abaxial epidermal cells as opposed to that in the adaxial epidermal and the palisade mesophyll cells (Figure 4.14). Preferential localization of Cr inside the hairy parts of the leaves (trichomes) has been observed in *L. tridentata* (Arteaga *et al.*, 2000). Corradi *et al.* (1993) also found that in the seedlings of *S. sclarea*, Cr accumulated in relatively large amounts in epidermal hairs of the cotyledons. Many hyperaccumulator plants are reported to sequester toxic metals in the vacuole of their epidermal cells (McGrath *et al.*, 2002). The ability of the leaves of *B. juncea* to accumulate Cr preferentially in the epidermis and lower spongy mesophyll might have increased the plant's tolerance to the metal by protecting photosynthesis, which takes place predominantly in palisade cells.

The X-ray image of a lateral root tissue (Figure 4.15) illustrates that Cr is mostly concentrated in the epidermis and cortex compared with the root vascular

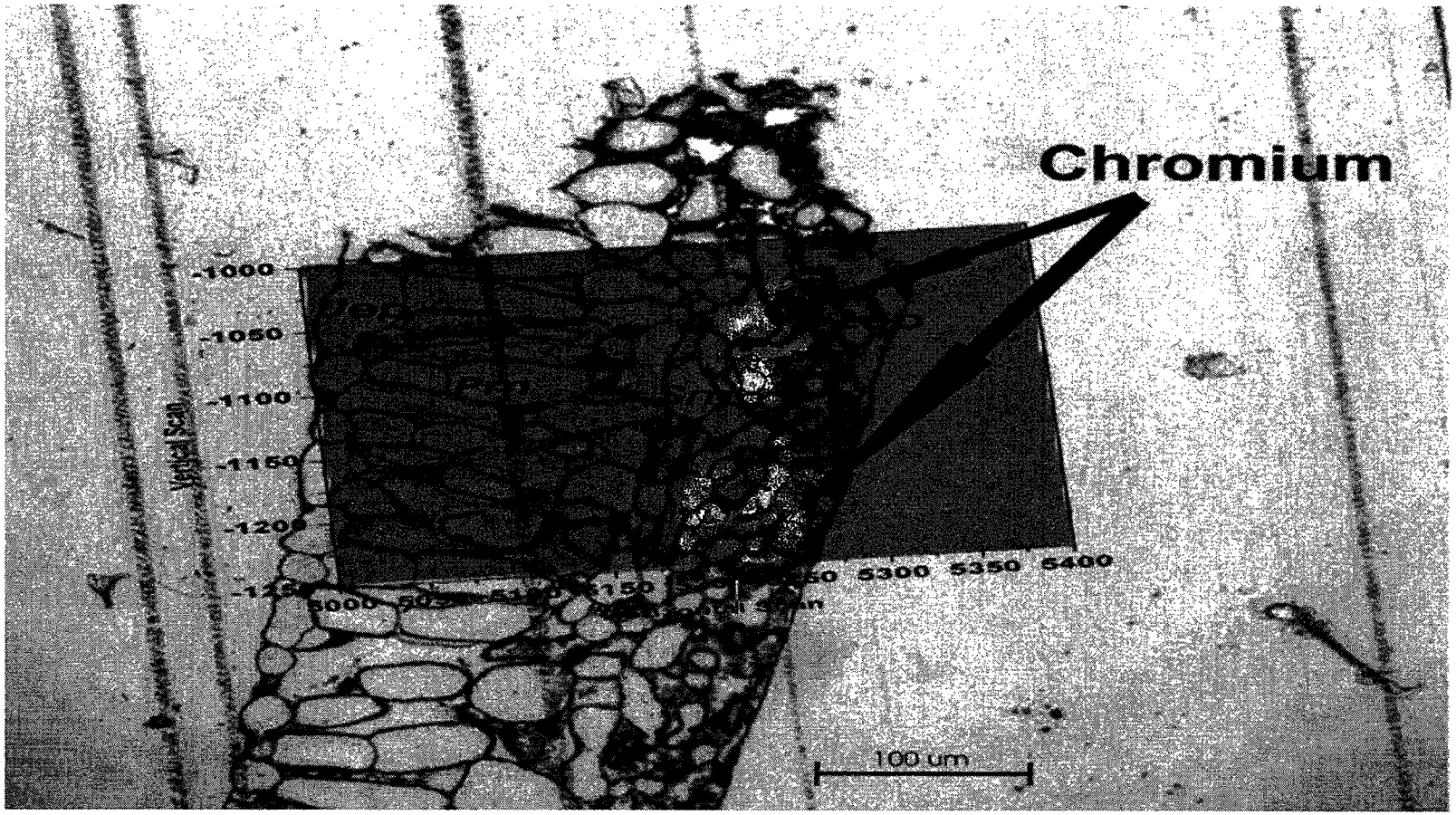

Figure 4.14. X-ray microprobe image (200x) of a cross-sectioned leaf of *Brassica juncea* grown for 69 days in field-moist soil treated with 100 mg kg^{-1} $CrCl_3 \cdot 6H_2O$ (***Uep*** = upper or adaxial epidermis, ***Lep*** = lower or abaxial epidermis, ***Pm*** = palisade mesophyll, ***Sm*** = spongy mesophyll). Note the accumulation of Cr (white color) in the lower spongy mesophyll and epidermis.

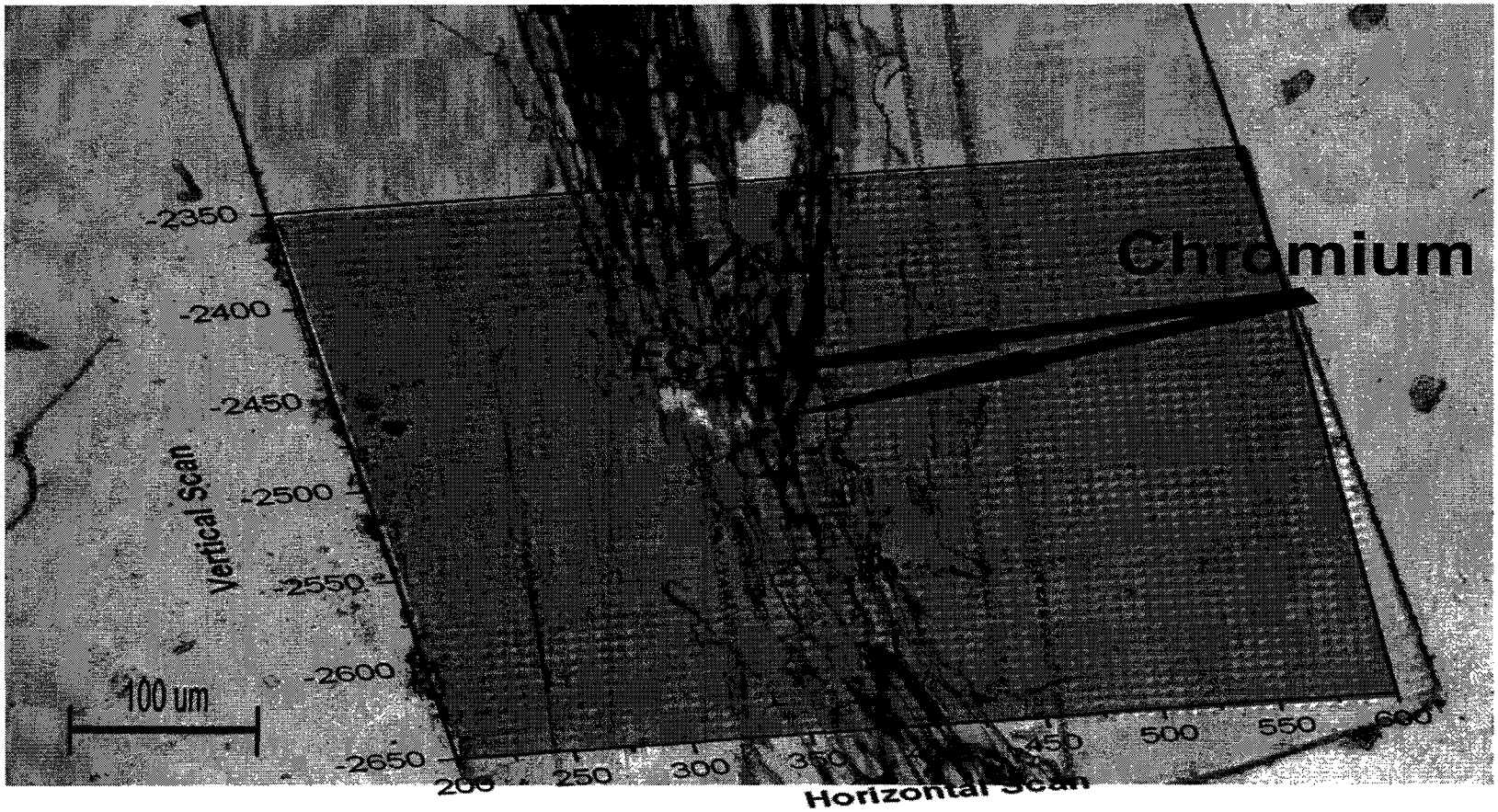

Figure 4.15. X-ray microprobe image (200x) of a longitudinal-sectioned lateral root of *Brassica juncea* grown for 69 days in field-moist soil treated with 100 mg kg^{-1} $CrCl_3 \cdot 6H_2O$ (***Ec*** = epidermis and cortex, ***Vs*** = vascular system: xylem and phloem). Note the greater accumulation of Cr (white color) in the interior epidermis and cortex compared with the xylem and phloem.

system (phloem and xylem). In general, due to the barrier in the endodermis, i.e., the Casparian strip, epidermal and cortical cells contain higher amount of metals than the cells in the vascular tissue (Barcelo and Poschenrieder, 1990). For example, Seregin and Ivanov (1997) showed that in longitudinally dissected roots, both Pb and Cd entered only one or two layers of the vascular system, while in the cortex, these ions moved across several layers as far as the endodermis. The Casparian strip is a lignified part of the primary cell wall consisting of cellulose and lignin (Schreiber *et al.*, 1994). The plasmalemmae of endodermal cells are very tightly bound to the Casparian strips so that even severe plasmolysis does not separate the endodermal protoplasts from the anticlinal cell walls in this area (Bonnett, 1968). Therefore, the movement of metal ions from the cortex into the central part of the root can only be symplastically across the plasma membranes of the cortical and endodermal cells. The frequent occurrence of plasmodesmata in the periclinal cell walls of the endodermis should also significantly facilitate the uptake of ions from the cortical symplast into the endodermal symplast (Clarkson, 1991).

Vazquez *et al.* (1987) observed both the injury (severely damaged epidermal cells) and the higher Cr content on the root surface of Cr (VI)-treated *P. vulgaris* compared with the plant cells in the central part of the root, which is in agreement with the present results. Further, Shewry and Peterson (1974) found that precipitation of Cr in vacuoles of root cortical cells, which generally contain relatively large vacuoles (Esau, 1977), may be responsible for the lower

injury observed in the central part of the root compared with the epidermal and cortical cells. Some Cr found in the vascular tissue of the lateral root of *B. juncea* (Figure 4.15) could have moved either symplastically to avoid the Casparian strip or apoplastically through the gaps in the endodermis of lateral roots.

5.0. CONCLUSIONS

The findings in the present study clearly illustrate the effects of the two forms of Cr on growth of *Brassica juncea* and soil microorganisms. In particular, both Cr (III) and Cr (VI) reduced plant root biomass and had a negative effect on root anatomical properties such as root and xylem diameters and number of large xylem cells and their width, although this effect was more pronounced in the tap than in the lateral roots. However, Cr (VI) seemed to affect these plant characteristics to a greater extent than Cr (III). Despite the fact that leaf cells were not affected by Cr (VI), this form of chromium was more toxic, causing mild leaf chlorosis, evident reduction in shoot biomass, increase in root exudation of low-molecular-weight organic acids, and damage of soil microorganisms, whereas Cr (III) did not appear to affect any of these plant and soil properties. Moreover, although neither Cr (III) nor Cr (VI) did inhibit shoot length, the latter treatment did have a negative impact on the girth of the plant stem and on the development of the vascular system, particularly the number of vascular bundles and xylem cells.

The differential response of microorganisms to Cr (III) and Cr (VI) could be related to contrasting bioavailability of the two Cr forms in the soils. Due to significantly higher amounts of soluble and, hence, bioavailable Cr (III) and Cr (VI) extracted from Cr (VI)-treated soils, soil microbial activity (C flush) was inhibited in this treatment. The differences in growth responses of *B. juncea* to

Cr (III) and Cr (VI) suggest that the metal absorption and transport within the plant may be considerably different for these two Cr species. In particular, consistent with the literature, *B. juncea* concentrated Cr mainly in the roots, while Cr translocation to the shoots was generally low. Moreover, although in the roots, a similar amount of Cr was accumulated from both Cr (III) and Cr (VI) treatments, more Cr was translocated to the plant shoots in the latter treatment than in the former.

Although there were observed injuries in the roots and shoots, *B. juncea* was tolerant of Cr at the concentrations used in the present study. Furthermore, the XANES analyses detected Cr present mostly as Cr (III)-acetate and Cr (III)-oxalate, in the roots and leaves of the Cr (VI)-treated plants, respectively. This demonstrates the ability of *B. juncea* to convert more toxic Cr (VI) to less toxic Cr (III) and indicates the important role of organic ligands, most likely originating from plant root and/or microbial exudates, in plant survival under Cr stress. Additionally, the several sites of Cr localization at the cellular levels were identified as metal deposition, and probably sequestration, in epidermal and cortical cells in the roots and epidermal and spongy mesophyll cells in the leaves, most likely in the vacuoles.

In summary, despite the fact that, under greenhouse conditions, *B. juncea* did not seem to remove much Cr from the soils (0.3 and 0.4% from the Cr (III)

and Cr (VI) treatments, respectively), the ability of the plant to tolerate and reduce Cr (VI) to Cr (III) could make it a potential candidate for phytostabilization.

6.0. FUTURE STUDIES AND RECOMMENDATIONS

Considering limited Cr bioavailability and low plant uptake, it appears that the type of soil used in this experiment does not pose significant risk to the environment. Nevertheless, to estimate biovailability and consequently resolve the fate and threat of soil Cr, the mass balance studies accounting for leached, exchangeable, organically-bound, Fe/Mn oxide-bound and residual concentrations of the added metal are needed. Moreover, the conditions of the Cr-spiked soils used in this greenhouse study might not reflect real conditions in field soils, either naturally or anthropogenically contaminated (aged) with Cr, where complex interactions with several other metals are likely. Therefore, field work is required to receive an accurate assessment of the potential of *Brassica juncea* for remediation of soil Cr. Future research should focus on soil characteristics such as pH, organic matter, moisture, and mineralogy (clay minerals, Fe and Al oxides), which are crucial in Cr speciation and behavior in soil and, as a result, in plants. The investigation of the effects of higher Cr concentrations, which could possibly induce metal uptake by *B. juncea*, should also be carried out to further elucidate the potential of the plant to tolerate and remediate soil Cr. Finally, more samples of root, leaf, and stem tissues should be collected in order to obtain more representative data on Cr distribution in the plant.

7.0. REFERENCES

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APPENDIX

Table 1. Profile description of soil from Aleza Lake (Elevation 704 m; North 54°03.844'; West 122°02.473'); Glaciolacustrine parent material.

Horizon	*Depth (cm)*	*Description*
L	3.5-1.5	Needles, deciduous leaves, and twigs.
F	1.5-0	Dry; very dark grayish brown (10YR 3/2); structureless; loose; fibrous; abundant, fine to medium roots; common fauna; few earthworms, larvae; common, random mycelia; abrupt, smooth boundary; 0.5-2.5 cm thick.
Ahe	0-5.5	Grey (7.5YR 5/1 d); sand; weak, fine, granular; loose; plentiful, fine to medium roots; clear, smooth boundary; 1.5-7.0 cm thick.
Bf	5.5-30.5	Dark yellowish brown (10YR 3/6 m); sand; structureless; loose; plentiful, fine to medium roots; clear, smooth boundary; 25.5-28.5 cm thick.
Bfj	30.5-44	Dark yellowish brown (10YR 3/4 m); sand; structureless; very friable; plentiful, very fine roots; clear, smooth boundary; 19.0-43.5 cm thick.
BC	44-59.5	Dark brown (10YR 3/3 m); sand; structureless; very friable; few, medium roots; clear, smooth boundary; 16.5-28.0 cm thick.
C	59.5-81	Very dark grayish brown (10YR 3/2 m); sand; structureless; very friable; few, medium roots, abrupt, irregular boundary; 5.5-23.0 cm thick.
IIC	81+	Brown (10YR 4/2 m); silty clay; massive; firm; no roots; common, thin to moderately thick, on ped surfaces (7.5YR 4/4 m) clay films.