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P. Boccia [a], C. Meconi [a], M. Mecozzi [b] & E. Sturchio [a]
[a] Italian Workers' Compensation Authority (INAIL), Department of Production Plants and Anthropic Settlements (DIPIA), Rome, Italy
[b] Laboratory of Chemometrics and Environmental Applications, Rome, Italy

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MOLECULAR MODIFICATIONS INDUCED BY INORGANIC ARSENIC IN *Vicia faba* INVESTIGATED BY FTIR, FTNIR SPECTROSCOPY AND GENOTOXICITY TESTING

P. Boccia[1], C. Meconi[1], M. Mecozzi[2], E. Sturchio[1]

[1]Italian Workers' Compensation Authority (INAIL), Department of Production Plants and Anthropic Settlements (DIPIA), Rome, Italy
[2]Laboratory of Chemometrics and Environmental Applications, Rome, Italy

Exposure to inorganic arsenic (iAs) through drinking water is a major public health concern affecting most countries. Epidemiologic studies showed a significant association between consumption of iAs through drinking water and different types of cancer. However, the exact mechanisms underlying As-induced cancer and other diseases are not yet well understood. The aim of this study is to determine the effects of exposure iAs (20 or 30 mg/L) on *Vicia faba* seedlings in terms of phytotoxicity, genotoxicity, and spectroscopy by investigation of molecular modifications using infrared (FTIR) and near infrared (FTNIR) spectroscopy. Further, the mitigation effects of a precursor of glutathione (GSH), *N*-acetylcysteine (NAC), were also assessed. Spectroscopic and genotoxicity analysis demonstrated that specific molecular changes were directly correlated with iAs exposure. Comet assay in *Vicia faba* showed significant effects at concentrations of 20 and 30 mg/L, depending on the structural changes involving nucleic acids as identified by FTIR and FTNIR spectroscopy. Results of phytotoxicity and micronuclei tests were significant only at higher iAs concentrations (30 mg/L), where an antioxidant effect of NAC was noted. The two spectroscopic techniques demonstrated molecular modifications predominantly associated with chemical interactions of iAs with biomolecules such as nucleic acids, carbohydrates, lipids, and proteins in *Vicia faba*. Our findings suggest that further studies are required to better understand the mechanisms underlying toxicity produced by different As chemical forms in vegetal and agricultural species.

Arsenic (As) is a ubiquitous metalloid found in the atmosphere, soils, rocks, and waters. Arsenic is mobilized through a combination of natural processes, such as weathering reactions, biological activity, and volcanic emissions, and different anthropogenic activities such as mining and use of arsenical pesticides and herbicides (Smedley and Kinniburgh, 2002). Extensive chronic inorganic arsenic (iAs) poisoning is occurring in many countries, especially in South America and some regions of Asia, potentially affecting tens of millions of people, due to iAs contamination in the drinking water extracted from shallow underground aquifers (Lin et al., 1998; Zhao, 2010; Orloff et al., 2009). Following the accumulation of evidence for the chronic toxicological effects of iAs in drinking water, recommended and regulatory iAs limits of many authorities are being reduced (WHO, 1993). The WHO guideline value for iAs in drinking water was provisionally reduced in 1993 from 50 to 10 μg/L. Despite the presence of this limit, the concentrations of iAs detected in drinking water are greater than 10 μg/L in many countries globally (WHO, 2001). Acute As intoxication associated with the ingestion of well water containing high concentrations of 1.2 and 21 mg As/L was reported (Feinglass, 1973; Wagner, 1979). Inorganic arsenic is considered as class 1

Address correspondence to E. Sturchio, Italian Workers' Compensation Authority (INAIL), Department of Production Plants and Anthropic Settlements (DIPIA) Via Urbana, 167-00184 Rome, Italy. E-mail: e.sturchio@inail.it

"carcinogenic to humans" by the International Agency for Research on Cancer (IARC, 2004). Many of the human adverse health effects attributed to As showed a significant association between consumption of iAs through drinking water and cancers of the skin, lung, bladder, liver, and kidney, neurologic disease, cardiovascular disease, developmental disorders, and other nonmalignant diseases (Wagner et al., 1979; Tsai et al., 1998; Golub et al., 1998; Chiu et al., 2007; Klein et al., 2007; Chen, 2011). Several mechanisms by which arsenical compounds induce tumorigenesis were proposed, including oxidative stress, DNA repair alteration, chromosomal aberrations (CA), modification of cell proliferation, aberrations in gene expression, cocarcinogenesis with other environmental toxicants, and epigenetic mechanisms that alter DNA methylation (Bernstam and Nriagu, 2000; Ren et al., 2011). Thus, a correlation between iAs exposure, cytotoxicity and genotoxicity, mutagenicity, and tumor promotion was postulated (Klein et al., 2007).

The genotoxic effect of carcinogenic substances that induce DNA breaks may lead, if not correctly repaired, to propagation of mutations, and consequently neoplastic processes. It is possible that the oxidative stress induced by iAs aberrantly regulates expression and function of DNA repair genes, resulting in loss of the DNA repair capacity (Singh et al., 2011). In addition, iAs is known to induce chromosome/chromatid breaks or exchanges (clastogenicity), formation of apurinic/apyrimidinic sites, DNA protein cross-links, CA, and interaction with spindle function during mitosis or meiosis, generating chromosome segregational errors (nondisjunction and noncongression), leading to aneuploidy and/or polyploidy (aneugenicity) (Duquesnoy et al., 2010).

Exposure to As typically results from either oral consumption through contaminated drinking water, soil, and food or inhalation in an industrial work setting. Inorganic As is quickly absorbed after oral exposure by most mammalian species, primarily methylated in liver, and excreted in urine. The inorganic pentavalent arsenic (As^{V}) form is converted to the trivalent arsenic (As^{III}) form, with subsequent methylation to monomethylated (MMA) and dimethylated arsenicals (DMA) species. The main exposure route to iAs is consumption of contaminated food, especially seafood (i.e., fish) and cereals (i.e., rice) traditionally cultivated in flooding fields. Groundwater contaminated by iAs is often used in agriculture to irrigate crops, and iAs then find its way into grains of plants, such as rice and wheat, and into vegetables and fruit plants. This may potentially enable As to enter the human food chain (Beni et al., 2007). Different plant populations or species show wide variation in amounts of As that may accumulate, and distribution of accumulated As also differs with respect to root and shoot tissues levels.

The mechanisms underlying toxicity differ among iAs species and it is not fully understood in plants. Trivalent iA is more toxic then pentavalent iAs, as it (1) binds to sulfydryl groups of proteins affecting their structures or catalytic functions (Hughes 2002), (2) induces an upregulation of enzymes involved in the antioxidant responses (Mylona et al., 1998; Requejo and Tena, 2005), and (3) alters DNA repair mechanism (Gebel, 2001; Wu et al., 2010). Arsenite (As^{III}) enters plant root cells via aquaglyceroporin channels, represented by the nodulin 26-like intrinsic proteins (NIP) in higher plants, which are structurally and functionally similar to microbial and mammalian aquaglyceroporins (Wallace et al., 2006). Arsenate (As^{V}), as an analogous phosphate compound, (1) is taken up by plant roots via phosphate transporters, (2) interferes with cellular processes such as phosphorylation and ATP synthesis, (3) generates reactive oxygen species (ROS) in plant tissues, and (4) induces lipid peroxidation and DNA and oxidative base damage (Sharma et al., 2012).

The translocation system of iAs species from root to the aboveground parts of the plant is still not well defined, although several studies indicated that both roots and shoots reduce As^{V} to As^{III} (Duan et al., 2007), but roots may exhibit a major arsenate reduction activity because arsenite is the predominant form found in the xylem sap of a number of plant species (Zhao et al., 2010). Several studies demonstrated that

As transported in xylem sap occurs in several different species, such as As^{V}, As^{III}, MMA, and DMA, and probably the ratio of As^{III} to As^{V} could be plant dependent. In rice, it was noted that As^{III} is loaded into the xylem via the silicon transporter Lsi2, while the loading mechanism of As^{V} and methylated As species have not been identified until now (Ma et al., 2007; Kalle Uroic et al., 2012). In response to iAs, plants assimilate sulfate to form cysteine (Cys) for the synthesis of glutathione (GSH) in two ATP-dependent steps. Subsequently, plants induce synthesis of phytochelatins (PCn), the polymers of GSH, through the enzyme phytochelatin synthase (PCS). Phytochelatins are transported from root to shoot and vice versa. Before detoxification, As^{V} is reduced to As^{III} by arsenate reductase (AR) using GSH as a reducing agent. Phytochelatins and GSH coordinate trivalent As to form a variety of complexes. These complexes are sequestered in the vacuole by ABC-type transporters, the identity of which is not yet established (Zhao et al., 2010).

Another detoxification mechanism that is generally underinvestigated in plants involves iAs methylation according to the so-called "Challenger pathway." In fact, methylated iAs species, such as MMA, DMA, and trimethylarsine oxide (TMAO), were identified in plant samples. Zhao et al. (2010) provided evidence of iAs methylation in plants, although the plant As methyltransferase gene is yet to be identified. An additional possible constitutive mechanism of detoxification in plants, suggested recently by Logoteta et al. (2009) but not well known, is the efflux of arsenite to the external medium by As^{III} transporters against the concentration gradients or via acquaglycerporins down the concentration gradient (Dziubinska et al., 2012).

The aim of this study was to assess the potential protective effect of a precursor of GSH, *N*-acetylcysteine (NAC), that may increase iAs tolerance of plants exposed to acute iAs concentrations. The effects of iAs acute exposure on *Vicia faba* seedlings (Wu et al., 2010; Talukdar 2011) was determined using spectroscopic infrared (FTIR) and near infrared (FTNIR) techniques, as well as by phytotoxicity and genotoxicity testing. The potential mitigation effect of NAC was also studied. NAC is a general antioxidant able to increase cellular pools of free radical scavengers (Alscher, 1989) and for which the application was reported to decrease oxidative stress produced by herbicides (Iriti et al., 2009) and UV-B (Malanga et al., 1999).

MATERIALS AND METHODS

The experimental protocol consisted of in vitro phytotoxicity and genotoxicity tests and spectroscopic techniques on primary roots of *Vicia faba* grown in differentially polluted soils. *Vicia faba* seeds were sowed in 250 g quartz sand soil in aluminum basins. Each basin, containing 25 seeds, was treated with 40 ml sodium arsenate dibasic heptahydrate (20 or 30 mg/L As) and incubated in a climatic chamber (at 21 ± 1°C and 60% relative humidity) for 5 d to enable germination. Seeds grown in a quartz sand soil basin irrigated with 40 ml water were used as the negative control. A further test was performed to study the mitigation effect of NAC in genotoxicity tests with iAs at 30 mg/L and NAC 300 mg/L. All the experiments were repeated thrice.

In Vitro Phytotoxicity and Genotoxicity Testing

Phytotoxicity After 5 d seedlings were extracted and phytotoxicity was determined by measuring primary root length of *V. faba* seedlings exposed to iAs. The comparison of the mean of primary root length of each sample with that of the control provided an indication of adverse effects.

Genotoxicity Tests

Comet assay Single-cell gel electrophoresis (SCGE) is a cytogenetic test used to evaluate in a single cell genetic damage induced by mutagenic agents. The test is a simple, sensitive, and rapid short-term genotoxicity method for measuring under alkaline conditions, pH ≥ 13.5, all DNA structural conformation changes, including double-strand/single-strand (ds/ss) breaks, alkali-labile sites such abasic sites,

and oxidized bases (Koppen and Verschaeve, 1996; Menke et al., 2000). The test is performed under alkaline unwinding/alkaline electrophoresis (A/A) protocol (Angelis et al., 2000) as modified by Sturchio et al. (2011). Briefly, nuclei from chopped *V. faba* root tips are filtered and mixed with agarose and set on a microscopic slide for electrophoresis (300 mA, 25 V for 45 min). Comets are viewed with an epifluorescence microscope; image analysis is carried out using an interactive image analyzer (Komet 5.5). The specificity of the comet test may be enhanced to investigate specific types of DNA damage by adding DNA modifying enzymes. The Olive Tail Moment ([Tail.mean – Head.mean] × Tail%.DNA/100) is used as a parameter of DNA damage (μm). Tail moment is defined as the product of the tail length and the fraction of total DNA in the tail. Tail moment incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed/broken pieces (represented by the intensity of DNA in the tail).

Micronuclei test Micronuclei (MN) are Feulgen-positive corpuscles localized within the cell wall in the cytoplasmatic area surrounding the main nucleus. MN are formed by chromosome or chromosome fragments that are not incorporated into daughter nuclei at the time of cell division (Ma, 1982). The MN test is performed using root meristems of *Vicia faba*. The micronucleated cells frequency is scored from 15,000 cells (15 root tips, 1,000 cells for tip) (De Marco et al., 1990; De Simone et al., 1992).

Statistical Analysis

Each experimental data set was subjected to analysis of variance (ANOVA) followed by Dunnett's test for comparing the difference of mean and standard error at the .05 level for statistical significance versus control using the statistical software package SPSS (Chicago, IL).

Spectroscopic Analysis

For any spectroscopic measurement the primary roots of seedlings were cut off, freeze-dried, and then lyophilized.

FTIR Spectroscopy FTIR measurements were performed by a Jasco Fourier transform spectrophotometer model 410, equipped with a Pike Technology accessory for collecting spectra in diffuse reflectance mode, using a metal platform for placing samples. The instrument chamber was preheated to reduce atmospheric air interference depending on CO_2 mainly. Spectral acquisition is performed by means of 500 scans with 4 cm^{-1} of spectral resolution in the 650–4000 cm^{-1} range, using KBr as a spectroscopic blank. A cosine function is applied as apodization function. Spectra are baseline correct and submitted to a 15-point smoothing filter for noise reduction. Then any spectrum is normalized by means of the Amide I band at 1650 cm^{-1}. All the spectra are saved as ASCII files.

FTNIR Spectroscopy FTNIR spectra were collected by a Jasco Fourier transform spectrophotometer model 4200, equipped with a Pike Technology accessory for collecting spectra in diffuse reflectance mode. Spectra were taken using a metal platform for placing samples, after 500 scans at 4 cm^{-1} of spectral resolution in the 3800–10,000 cm^{-1} range, using KBr as spectroscopic blank. Spectra are baseline correct and submitted to the same 15-point smoothing filter for noise reduction already reported for FTIR spectra. Then spectra are normalized by means of the band at 4030 cm^{-1}. All the spectra are saved as ASCII files.

RESULTS

In Vitro Phytotoxicity and Genotoxicity Tests

Phytotoxicity testing showed statistically significant results at highest concentrations. The addition of NAC 300 mg/L improved the growth of roots co-incubated with iAs (Figure 1a). Comet assay in *Vicia faba* illustrated significant results at both 20 and 30 mg/L concentrations, in a concentration-dependent manner (Figures 1b, 2a, and 2b), and in accordance with structural changes involving nucleic acids identified with FTIR (Figure 3). Further,

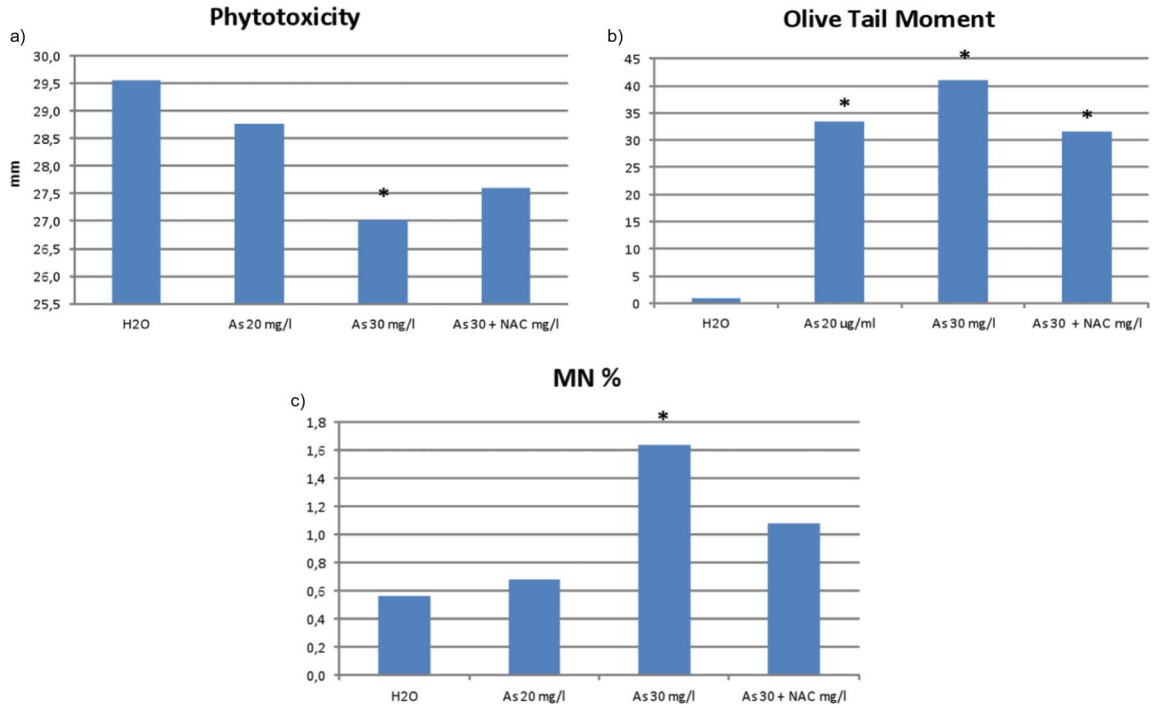

FIGURE 1. Genotoxicity and phytotoxicity tests. Asterisk indicates significant differences by ANOVA with Dunnett test at $p < .05$. (a) Phytotoxicity test. (b) Comet assay; Olive Tail Moment is used as parameter to measure DNA damage. (c) Micronuclei test (MN); the frequency of micronuclei is expressed as a percentage (color figure available online).

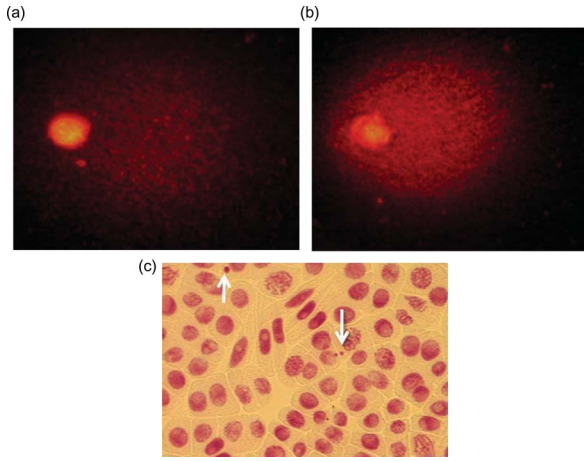

FIGURE 2. Comet assay images after iAs exposure at 20 mg/L (a) and 30 mg/L (b) and micronuclei formation after iAs 30 mg/L treatment (c) (color figure available online).

a clastogen effect of iAs was noted in the MN test only at the highest concentration (30 mg/L) without NAC present (Figures 1c and 2c). The antioxidant effect of NAC as shown by a reduction of MN frequency in *Vicia faba* cells occurred with iAs at 30 mg/L and NAC at 300 mg/L (Figure 1c).

Spectroscopic Analysis

FTIR spectra of lyophilized root meristems samples exposed to iAs are reported and compared with the related control samples (Figure 3). In normalized spectra, the observed intensity and peak shape changes represent quantitative and structural changes related to molecular modifications produced by iAs exposure. Polluted samples with iAs at 20 or 30 mg/L showed some intensity and shape changes compared to the control, mainly related to polysaccharide content detected by the bands at 1160 and 3350 cm^{-1}. Lipid content and structural changes related to vegetal and pigment compounds are illustrated by changes of the large band between 2000 and 2400 cm^{-1} and by alterations of aliphatic chains between 2800 and 2950 cm^{-1} and between 1350 and 1420 cm^{-1} bands. Other band shape changes were observed for the P=O group of nucleic acids between 1210 and 1250 cm^{-1}. Due to several functional groups being modified after exposure, it is conceivable

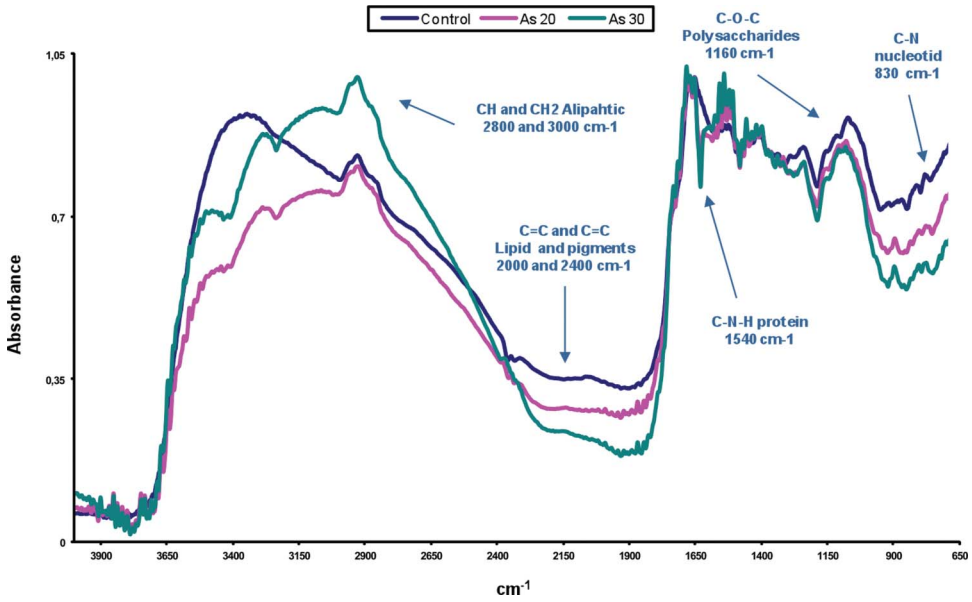

FIGURE 3. FTIR spectra of lyophilized root meristems samples exposed to iAs (20 or 30 mg/L). The arrows show the most significant molecular changes observed (see spectroscopic result section) (color figure available online).

that iAs produced both quantitative and structural changes involving carbohydrates, proteins, lipids, and nucleic acids.

FTNIR spectra showed additional information concerning structural modifications produiced by iAs exposure (Figure 4). In fact, the most evident modifications detected by FTNIR spectra included both tested iAs concentrations, demonstrating a modified shape of the first overtone –OH group between 6400 and 6900 cm^{-1}, assigned to carbohydrates and proteins. Other bands showed structural changes related to hydrogen bond interactions and those belonging to both inter- and intramolecular hydrogen bonds. Intermolecular hydrogen bond changes were evidenced by the interaction band between the –OH group of carbohydrate and the –C=O group of proteins, located in the 5100–5200 cm^{-1} range. Intramolecular hydrogen bond alterations are those involving the –C=O and –NH group of proteins, detected by the interaction FTNIR band in the 4500 and 4900 cm^{-1} range.

DISCUSSION

Spectroscopic and genotoxicity analysis showed that specific molecular changes were directly related to acute iAs exposure. The chemical behavior of As is similar to that of phosphorus in soil where arsenate is taken up via the phosphate transport systems, and subsequently acts in the cell as a phosphate analog to disrupt phosphate metabolism, inhibit DNA repair and induce MN formation (Wu et al., 2010). In particular, this mechanism might result in replacing phosphate with iAs in the phosphate groups of DNA, and the changes shown with FTIR spectra analysis by the P=O group of nucleic acids between 1210 and 1250 cm^{-1} are associated with this partial substitution reaction. DNA damage is clearly shown by the significant results of comet assay at both iAs concentrations. It is known that As-induced ROS, such as superoxide, hydrogen peroxide, and hydroxyl radicals, attack proteins, lipids, and deoxyribose, purine and pyrimidine bases in DNA molecule, producing DNA strand breaks, which increase the probability of chromosome/chromatid fragmentation, thereby leading to MN formation (Wu et al., 2010). DNA damage, evidenced by comet assay and MN test, rose with increasing arsenate concentrations, indicating a genotoxicity effect of arsenate. Comet assay illustrated significant results at both 20 and

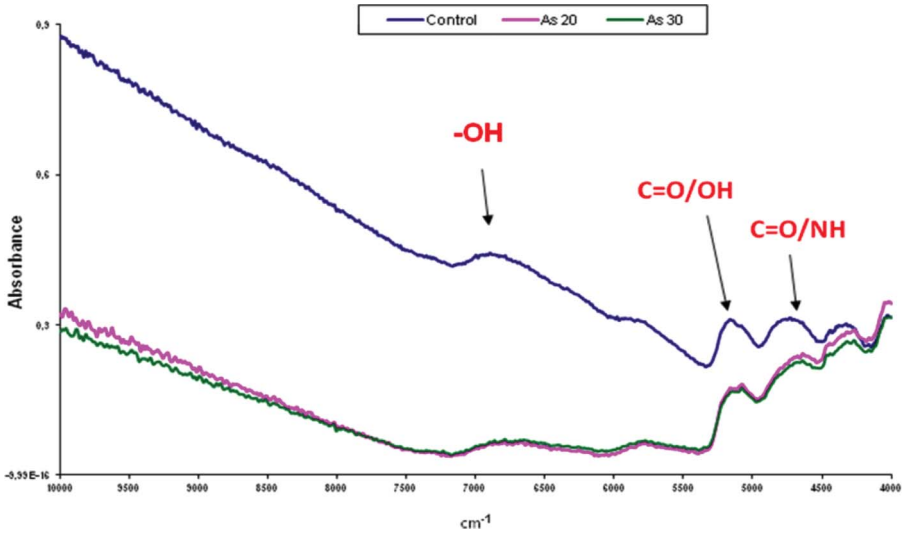

FIGURE 4. FTNIR spectra of lyophilized root meristems samples exposed to iAs (20 or 30 mg/L). The arrows show the most significant molecular changes observed (see spectroscopic result section) (color figure available online).

30 mg/L concentrations, in a concentration-dependent manner, in accordance with structural changes involving nucleic acids identified by FTIR analysis.

Cytoskeleton is an important cellular target of As-induced toxicity (Binet et al., 2006), and iAs may induce chromosomal lagging by interfering with microtubule assembly and spindle formation (Wu et al., 2010), leading to MN formation. In our experiment the clastogen effect of iAs was shown only at the highest concentration (30 mg/L). Further, FTIR analysis on *Vicia faba* indicated how iAs exposure may be related to structural changes of molecules, such as the lipid, and vegetal compounds. These biological macromolecules may be affected by ROS production resulting in lipid peroxidation and membrane damage (Sharma et al., 2012). FTNIR spectra showed additional information concerning structural changes related to proteins and polysaccharides. The modified shapes of the intramolecular hydrogen bond suggested changes in the secondary protein structure of *Vicia faba* involving the α helix to β secondary (i.e., sheet and turn) structure ratios. This hypothesis needs further study to be verified. In any case, these results confirm the molecular modifications observed by means of FTIR spectra because FTIR and FTNIR spectroscopy have already demonstrated their combined power in detecting structural changes in protein and polysaccharide contents of vegetal organisms (Mecozzi et al., 2011).

To minimize the harmful effects of ROS, plants have evolved an effective scavenging system based on antioxidant molecules and enzymes development in order to reduce xenobiotic-induced toxicity (Sheppard, 1992; Mehargh, 1994). In our experiment, phytotoxicity and genotoxicity effects were reduced by NAC treatment, confirming that NAC protects plants from oxidative stress damage and increases tolerance to iAs.

In conclusion, data indicate by FTIR, FTNIR, and genotoxicity testing that iAs induced molecular modifications mainly in carbohydrates, lipids, nucleic acids, and proteins. In addition, the application of the NAC confirms its property to increase resistance or tolerance of the plant subjected to environmental stress.

More studies are needed to better understand the mechanisms underlying toxicity of the different As chemical forms and how compartmentalization occurs in roots, in addition to some chemical and physiological conditions that may influence As phytoavailability (Meharg, 1994).

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