1993

Distribution and Intracellular Localization of Titanium in Plants After Titanium Treatment

G. Kelemen

A. Keresztes

E. Bacsy

M. Feher

P. Fodor

See next page for additional authors

Utah State University
MERRILL-CAZIER LIBRARY

Distribution and Intracellular Localization of Titanium in Plants After Titanium Treatment

Authors

G. Kelemen, A. Keresztes, E. Bacsy, M. Feher, P. Fodor, and I. Pais

DISTRIBUTION AND INTRACELLULAR LOCALIZATION OF TITANIUM IN PLANTS AFTER TITANIUM TREATMENT

G. Kelemen[1], Á. Keresztes[2, *], E. Bácsy[3], M. Fehér[4], P. Fodor[4], I. Pais[4]

[1]Department of Plant Physiology, [2]Department of Plant Anatomy,
Eötvös Loránd University, Budapest
[3]Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest
[4]Dept. of Chemistry and Biochemistry, University for Horticulture and Food Industry, Budapest
Hungary

Abstract

The physiological role of titanium in plants has not been elucidated yet, but a higher rate of growth, greater chlorophyll content and higher productivity, among others, may be attributed to this element. We have investigated the distribution of titanium in wheat seedlings after Titavit (a Ti-ascorbate containing plant conditioner) treatment applied either through the leaf or through the root. In field experiments, we also sprayed spinach plants with Titavit. We have found a practically unidirectional translocation of Ti from the shoot into the root, and a preferential localization of Ti in the nuclear cell fraction as seen by ICP-AES (inductively coupled plasma atomic emission spectrometry). Electron microscopic X-ray microanalysis after chemical fixation showed little or no accumulation of Ti in the cells of the treated organs. However, if there was an appreciable X-ray emission at 4.5 keV (Ti), it was recorded from the nucleoplasm and nucleolus. The comparison of ICP-AES and X-ray analyses leads us to the conclusion that the majority of Ti may be in a diffusible form in the treated cells, except the nuclei, where Ti may be bound firmly.

Key Words: Titanium, wheat, spinach, cell fractionation, inductively coupled plasma atomic emission spectrometry (ICP-AES), electron microscopy, X-ray microanalysis, nucleus, metal accumulation.

---

*Address for correspondence:
Á. Keresztes,
Dept.of Plant Anatomy, Eötvös L. University,
Puskin u.11-13, H-1088 Budapest, Hungary

Introduction

Titanium applied in water-soluble form considerably increased the yield of different crop plants, vegetables and fruits (Pais et al., 1979; Fehér et al., 1980; Pais, 1983; Fehér et al., 1984). It increased chlorophyll content in bean, and increased dry weight and the uptake of major and trace elements in bean and tomato (Ram et al., 1983, 1988). It enhanced the growth of tobacco callus and compensated for indoleacetic acid and kinetin deficiency of the culture medium (Maróti et al., 1984).

The mode of action of Ti in plants, however, is practically unknown (Dumon and Ernst, 1988). As a step in the direction of elucidating the effects of Ti, we carried out cell fractionation followed by inductively coupled plasma atomic emission spectrometry (ICP-AES) analysis, and electron microscopic X-ray microanalysis to detect intracellular localization of Ti.

Material and Methods

Seeds of *Triticum aestivum* L. were surface-sterilized by 3 % H_2O_2 for 10 minutes, then rinsed in running tap water for 1 hour and bubbled by air during the night. The seedlings were pregerminated in the dark for 2 days, after this in the light at 25°C for 9 days. As nutrient solutions, we used 0.125 mM $CaSO_4$ for the control plants and the same supplemented with 0.5 ppm Titavit (a Ti-ascorbate containing plant conditioner patented by Pais and Fehér, 1977) for the group treated through the root. Another treatment was spraying 5 ppm Titavit on the leaves of plants grown in $CaSO_4$ solution. The nutrient solutions were changed every three days. Other details of the treatments are given at Figs. 1-4.

Before homogenization, shoots and roots were thoroughly washed. In the case of shoots, the procedure described by Fehér (1987) was followed including rinses in tap water, 1:1 mixture of 0.1% Ultra detergent and 1% HCl, tap water, distilled water, then deionized water. As shown by the cited work, after this procedure, the last change of deionized water contained no appreciable amounts of Ti. In the case of roots, we omitted the detergent-HCl mixture.

The cell fractions from roots and shoots were obtained by homogenization with a Waring blender in a

buffer containing 300 mM sucrose, 5 mM MgCl and 50 mM Tris-HCl, pH 8 (1w/3v), filtered through 3 layers of Miracloth, and centrifuged by 150 g for 20 minutes. The pellet was washed three times. The fractions were checked by light microscopy and the pellet seemed to be rich in nuclei. The Ti content of pellet and supernatant after acidic digestion was measured by ICP-AES (Jarrel-Ash 61 type equipment).

In the field experiments, the leaves of *Spinacia oleracea* L. were sprayed with 5 ppm Titavit and sampled 4 days later. Samples from wheat roots and leaves, and spinach leaves washed according to the above described procedures were fixed in glutaraldehyde (5% v/v in 0.035 M K-Na phosphate buffer, pH 7.2), dehydrated in ethanol series and embedded in Durcupan ACM (Fluka). Semi-thin sections were mounted on copper grids and evaporated with carbon on both sides, then examined in a JEOL TEMSCAN 100CX analytical electron microscope operated at 80 keV, using the scanning transmission electron microscopy (STEM) mode and a specimen tilt of 35°. The X-ray spectra were recorded by an ORTEC energy-dispersive X-ray (EDX) analyzer.

For checking the washing procedure, we examined the surface of air-dried spinach leaf segments by EDX in the scanning mode and found no Ti.

Results

The uptake and distribution of Ti-ascorbate by the plants depended on the method of nutrition supply. When sprayed onto the leaf surface, Ti distributed almost equally between the leaves and roots (Figs. 1 and 2, treatment B). If administered through the roots, the majority of Ti taken up remained in the roots, while a relatively small amount was translocated into the leaves (Figs. 1 and 2, treatment F). Although the Ti uptake increased from the first to the third 3-day period in the roots (probably due to the growth), such an increase in Ti content was not shown by the shoot (Figs. 1 and 2, treatments C, D, E).

The intracellular Ti distribution was examined after differential centrifugation. The Ti content of the root homogenate fractions in the case of leaf spraying was equally distributed between the supernatant and the pellet. However, after the nutrition was supplied via roots, the majority of Ti was in the supernatant with a smaller quantity in the pellet (Fig. 3.)

The accumulation of Ti in the pellet from the sprayed leaves was high, as shown in Fig. 4. The fractions were examined by light microscopy, and the pellet was found to be rich in nuclei.

We wished to see whether a similar localization pattern can be obtained by an *in situ* method, the electron microscopic EDX microanalysis. We analyzed epidermal, mesophyll and vascular cells in leaves, and cells of the epidermis, cortex, endodermis, pericycle, vascular parenchyma and the tracheary cell wall in roots. After examining wheat samples, we could not detect significant Ti-peaks (at 4.5 keV in the X-ray spectra) in any compartment of the cells either in the treated roots, or

in the sprayed leaves. In sprayed spinach leaves, however, small but constant elevations appeared at or near 4.5 keV in the spectra of the nucleoplasm and the nucleolus, but not in those of other cell compartments (Figs. 5 and 6). The peak to background ratios reached 2.16 and 2.27 in the nucleoplasm and nucleolus respectively, while they varied between 1.10 and 1.58 in the control. (We defined the peak region from 4.36 to 4.64 keV and the background from 5.52 to 5.80 keV).

As we fixed the samples chemically, the solved or loosely bound forms of Ti were likely to be removed from the cells. Therefore, the results only say that if there is an appreciable amount of firmly bound Ti in the cell, it is within the nucleus.

Discussion

Several metals have been shown to accumulate within the nucleus once having entered the cell, e.g., aluminium (Matsumoto *et al.*, 1976; Morimura *et al.* 1978), zinc (De Filippis and Pallaghy, 1975); and mercury (De Filippis and Pallaghy, 1975; De Filippis, 1978).

According to our findings, this applies also to titanium. This is in agreement with the work of Köpf-Maier and Krahl (1983). By electron energy loss spectroscopy, they found titanium exclusively in nuclei of Ehrlich ascites tumor cells treated with titanocene dichloride. This compound also proved to have an antitumor effect. Our results seemingly are in contradiction with those of Nautsch-Laufer (1974) who concluded that Ti was located mostly in the cell walls of bean root cells, or maize root, stem and leaf cells. However, it is evident from the methodological chapter of the cited work that cell wall pieces were mixed with nuclei. Unfortunately, instead of this more complex description, the simple "cell wall" fraction was adopted by the literature (Dumon and Ernst, 1988).

The comparison of ICP-AES measurements from cell fractions and EDX analysis on chemically fixed sections shows that the majority of Ti was soluble or loosely bound. It remains an open question whether this part or the strongly bound (intranuclear) part of the cellular Ti content could be relevant to the effects mentioned in the **Introduction**, and how.

Our failure to detect Ti by EDX microanalysis in wheat leaf after foliar spray, in contrast to spinach leaf, may be in connection with the difference in leaf surfaces and/or with the different nutrition of these plants. In the wheat seedlings grown on $CaSO_4$ solution, nutrient deficiencies and imbalances could develop which could interfere with Ti uptake.

In roots, the Ti content on the dry weight basis was about 2 orders of magnitude higher than that in leaves (Figs. 3 and 4, the homogenates). Even if we take into account that the majority of this Ti content goes with the supernatant in case of roots, in contrast to leaves, it is still not evident, why we got no Ti signal from root cell nuclei, while we got some from leaf cell nuclei.

Localization of titanium in plants after titanium treatment
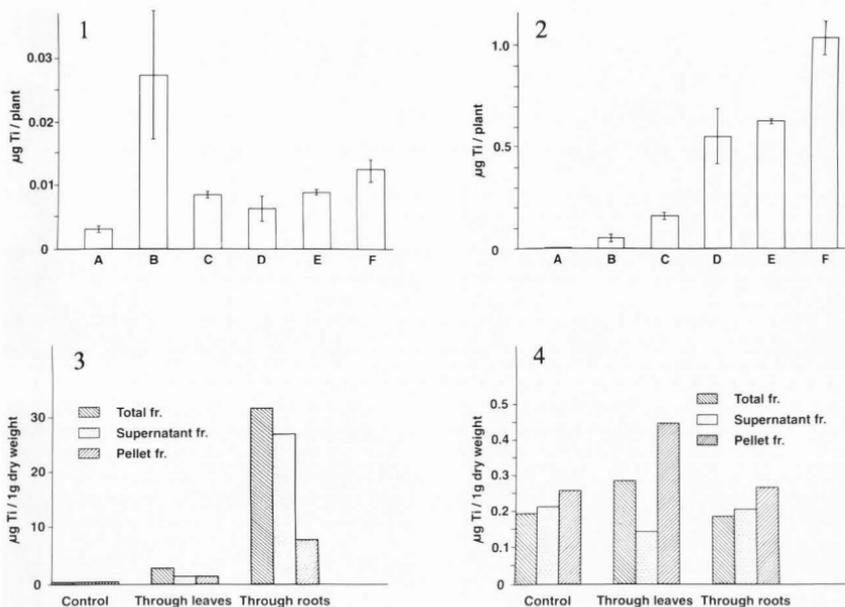
Figure 1. Ti content in the 9 days seedling leaves, depending on the method and sequence of Titavit-treatment. **A** control; **B** the leaves sprayed twice with 5 ppm Titavit before second and third nutrient solutions were exchanged; **C** plants grown in 0.5 ppm Titavit between 1-3 days; **D** 3-6 days; **E** 6-9 days; and **F** 1-9 days.

Figure 2. Ti content in the 9 days seedling roots depending on the method and sequence of Titavit treatment. Groups A-F are the same as in Fig. 1.

Figure 3. Ti content in the root homogenate fractions of 9 day seedlings treated by spraying the leaves twice with 5 ppm Titavit, or growing the plants in 0.5 ppm Titavit. Here and in the next caption, total fraction means the homogenate, and the Ti quantities are related to the dry weight units of the homogenate, the supernatant and the pellet.

Figure 4. Ti content in the leaf homogenate fractions of 9 day seedlings treated the same way as in Fig. 3.

In the treatment of plants (especially foods) with Ti, it is an important question, what further effects of Ti can be experienced in the food chain. Mixing Ti-ascorbate into the feed of different domesticated animals never resulted in toxic symptoms, but it helped body weight gain and health condition (Bokori *et al.*, 1985; Kimura *et al.*, 1985; Pais and Bokori, 1985). Some results showed that Ti-ascorbate could beneficially affect also animal reproduction (Pais *et al.*, 1989).

References

Bokori J, Nagy B, Pais I (1985). Application of titanium-ascorbate in animal feeding. In: Proc. 1st Internat. Trace Element Symp., Pais I. (ed), Univ. of Horticulture and Food Industry, Budapest, Hungary, 212-221.
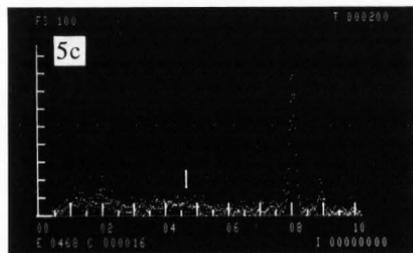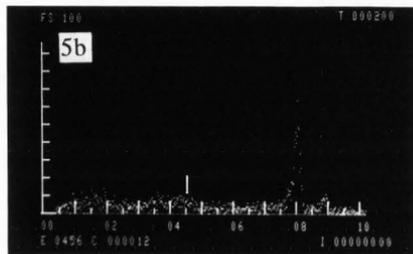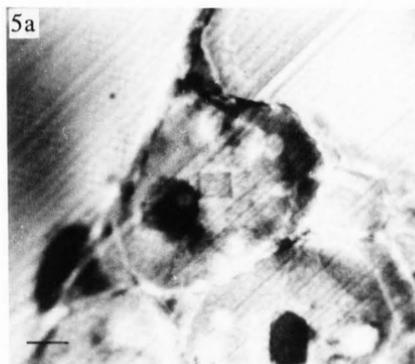
Figure 5. Vascular parenchyma cells in unstained semi-thin section from a sprayed spinach leaf examined by STEM (a). Rectangles in the nucleoplasm and nucleolus show the sites of analysis. Bar = 1 μm. In the X-ray spectrum of the nucleoplasm (b), small peaks representing Si (1.6 keV), P (2.0 keV), S (2.3 keV), Ca (3.7 keV) and Ti (4.5 keV) are seen. In the X-ray spectrum of the nucleolus (c), emission from P, S, Ca and Ti are found. Non-specific peaks: Cu (from the grid) at 8.0 and 8.9 keV.
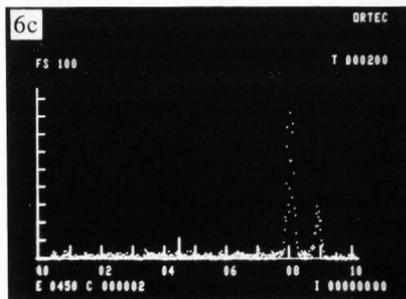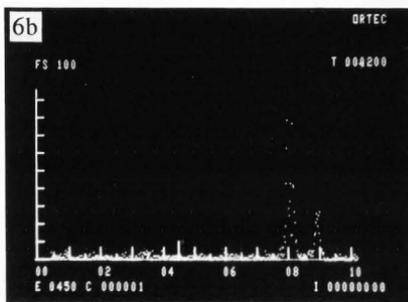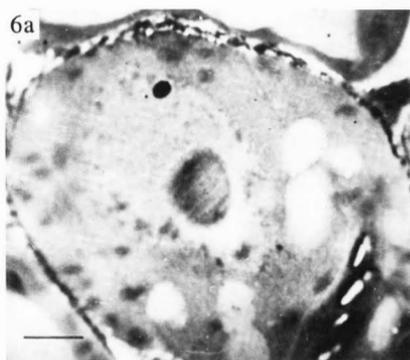
Figure 6. Vascular parenchyma cells in unstained semi-thin section from a control spinach leaf examined by STEM (a). Bar = 1 μm. In the X-ray spectra of the nucleoplasm (b) and nucleolus (c) there are no specific peaks.

70

De Filippis LF (1978). Localization of organo-mercurials in plant cells. Z. Pflanzenphysiol. **88**, 133-146.

De Filippis LF, Pallaghy CK (1975). Localization of zinc and mercury in plant cells. Micron **6**, 111-120.

Dumon JC, Ernst WHO (1988). Titanium in plants. J. Plant Physiol. **133**, 203-209.

Fehér M (1987). Effect of titanium on the productivity and biochemical processes of tomato plants (in Hungarian), C.Sc. Thesis, Library of Hung. Acad. Sci., Budapest.

Fehér M, Kádár K, Szabó Z, Gombkötő G, Lohonyai O, Pais I (1980). The role of titanium in plant life II. Foliar nutrition of alfalfa with titanium solution. Acta Agron. Acad. Sci. Hung. **29**, 71-76.

Fehér M, Lukács P, Vámos-Vigyázó L, Pais I (1984). Role of titanium in the life of plants IV. Effect of titanium on the germinative ability of wheat, maize and sunflower seeds and on the growth of seedlings. Acta Agron. Acad. Sci. Hung. **33**, 95-100.

Kimura S, Kawamura M, Shudo N, Pais I (1985). Nutritional studies on titanium-ascorbate. In: Proc. 1st Internat. Trace Element Symp., Pais I. (ed), Univ. of Horticulture and Food Industry, Budapest, Hungary, 239-249.

Köpf-Maier P, Krahl D (1983). Tumor inhibition by metallocenes: ultrastructural localization of titanium and vanadium in treated tumor cells by electron energy loss spectroscopy. Chem.-Biol. Interactions **44**, 317-328.

Maróti M, Pais I, Bognár J (1984). The role of titanium in plant life V. The effect of titanium on the growth of tobacco callus. Acta Agron. Acad. Sci. Hung. **33**, 315-322.

Matsumoto H, Hirasawa E, Torikai H, Takahashi E (1976). Localization of absorbed aluminium in pea root and its binding to nucleic acids. Plant and Cell Physiol. **17**, 127-137.

Morimura S, Takahashi E, Matsumoto H (1978). Association of aluminium with nuclei and inhibition of cell division in onion (*Allium cepa*) roots. Z. Pflanzenphysiol. **88**, 395-401.

Nautsch-Laufer C (1974). Die Wirkung von Titan auf den Stoffwechsel von *Phaseolus vulgaris* und *Zea mays* (The effect of titanium on the metabolism of *Phaseolus vulgaris* and *Zea mays*). Doct. Diss. Univ. Münster. Germany (in German).

Pais I (1983). The biological importance of titanium. J. Plant. Nutr. **6**, 3-131.

Pais I, Bokori J (1985). Application of titanium-ascorbate in animal feeding. Newsletter of FAO, Eur. Cooperative Network on Trace Elements. The Coordination Center, State Univ., Gent, Belgium. 69-74.

Pais I, Bokori J, Nagy B, Szilágyi M, Fekete S, Kolonics F (1989). The role of titanium-ascorbate in animal nutrition and reproduction. In: Proc. 6th Internat. Trace Element Symp., Anke M. Baumann W, Bräunlich H, Brückner C, Groppel B, Grun M (eds.). Verlage Abteilung der Friedrich Schiller Univ. Jena, Germany, 1356-1360.

Pais I, Fehér M (1977). Microelement-containing spray to increase the value components, particularly the sugar content in the fruit of horticultural and agricultural plants. Hungarian Patent, A 01 N5/100.

Pais I, Fehér M, Farkas E (1979). Role of titanium in the life of plants I. Acta Agron. Acad. Sci. Hung. **28**, 378-383.

Ram N, Verloo M, Cottenie A (1983). Response of bean to foliar spray of titanium. Plant and Soil **73**, 285-290.

Ram N, Verloo M, Cottenie A (1988). Influence of foliar and soil-applied titanium on tomato. Acta Agron. Hung. **37**, 43-46.

Discussion with Reviewers

L.J. Veto: How did the soil type, pH and temperature in your field experiments influence the availability and the accumulation of titanium in spinach plants?

Authors: As it is usual with most soil types [except the very acidic sandy soil, Ernst (1985)], Ti was not available in appreciable amounts for our plants, as shown by the X-ray spectra of the control. Nautsch-Laufer (1974, text reference) also failed to detect Ti in leaves of 5 plant species grown on peat or brown earth, although these soils were of pH 2.7 or 3.0, respectively, and she analyzed the leaves at the end of the vegetation period. Tonkonozhenko and Khlyupina (1974) measured Ti content in various soils and the alfalfa plants in the Krasnodar territory, and found no correlation.

W.H.O. Ernst: The authors state that this pellet was rich in nuclei? Where are the cell walls (debris), which are sedimented at 150 g?

L.J. Veto: You mention that the pellet seemed to be rich in nuclei, which has been checked by light micros-copy. What other evidence do you have that the pellet which was measured by ICP was "free" from other cell fragments (cell wall, vacuoles, chloroplasts, etc.)?

Authors: Most of the cell wall fragments were with-drawn by the filtering, the vacuoles ruptured during homogenization with their content contributing to the su-pernatant, the plastids and smaller organelles were not pelleted by 150 g.

W.H.O. Ernst: What is the detection limit of Ti in the samples by ICP-AES?

Authors: The detection limit of Ti in the samples by ICP-AES is 0.1 μg/g (Fehér, 1987, text reference).

L.J. Veto: Have you determined the presence and/or quantities of minor and/or trace elements by STEM-EDX?

Authors: From the micronutrients, Cl, and from the macronutrients, Mg and K, were found in both root and leaf samples, in addition to those mentioned in Fig. 5.

L.J. Veto: Although you are using semi-thin sections

for EDX-analysis, hence the electron interaction volume is "cut off", which improves your analytical resolution, what is your: **a)** X-ray count rate, **b)** spatial resolution, **c)** minimum detectable mass limit, and **d)** scanned area size of detection?

Authors: **a)** Typically 10-20 X-ray counts per seconds are representative. **b)** Approximately 20 nm spatial resolution, the diameter of scanning spot being between 10 and 20 nm. **c)** We have checked our apparatus with Al foils of different thickness, and 2×10^{-15}g yielded a definite peak. Therefore, the minimal detectable mass limit can be between this value and the theoretical minimum. **d)** The scanned area of detection varied from 0.2 to 2 μm^2.

L.J. Veto: You have used Durcupan ACM (Fluka) to embed your tissue and the semi-thin sections were examined by EDX. Can the embedding protocol cause elemental conflicts by leeching and ion dilution?

Authors: The embedding procedure could contribute to the leeching out, but we think, the loss occurred mainly during fixation and dehydration (although Ti bound to macromolecules could be retained during these procedures). This problem was investigated in detail by De Filippis and Pallaghy (1975, text reference) and De Filippis (1978, text reference). After inorganic Hg treatment, they found a major loss in Hg content during glutaraldehyde fixation while after organomercurial treatment, an additional marked decrease was recorded during dehydration. Resins had minimal effect.

L.J. Veto: In Figures 6b and 6c, you mention that in the X-ray spectra of the nucleoplasm and in the nucleolus there are no specific peaks of Si (1.740 keV), P (2.015 keV), S (2.308 keV) and Ca (3.691 keV). What is the explanation for this?

Authors: The reason may be a general leeching out, which at least partially could be counteracted in the treated plants by an enhanced uptake of some ions (Pais *et al.*, 1979; and Ram *et al.*, 1988; text references).

L.J. Veto: You have used a nutrient solution, supplemented with 0.5 ppm Titavit with seedlings and in the field experiments the leaves of spinach were sprayed with 5 ppm Titavit. Can you detect these low concentrations of Titavit with your X-ray analytical system?

Authors: Of course, we did not expect to detect 0.5 or 5 ppm Titavit by EDX. The basis of our analytical EM work was the supposition (supported by our ICP-AES analyses) that Ti could accumulate in certain cell compartments.

M. Verloo: What is the scientific relevance of the electron microscopic X-ray analysis?

Authors: EDX is an *in situ* method, in contrast to cell fractionation, which (especially in plants) has inherent drawbacks, like adhesion to or leakage from the organelles, etc. On the other hand, intranuclear distribution could be investigated exclusively by this method.

Additional References

Ernst WHO (1985). Bedeutung einer veränderten Mineralstoffverfügbarkeit (Schwermetalle, Al, Ti) für Wachstums- und Selektionsprozesse in Wäldern [The significance of a changed availability (heavy metals, Al, Ti) for growth and selection processes in woods]. Bielefelder Ökol. Beitr. **1**, 143-158 (in German).

Tonkonozhenko EV, Khlyupina MI (1974). Titanium in soils and plants of Krasnodar territory (in Russian). Pochvovedenie **3**, 38-45.