

Uptake of Uranium by Callus Cultures of Two Acacia Species

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ABSTRACT

This study aimed to use callus cultures of Acacia trees as indicator for the validity of employing these trees in the phytoremediation programs that were used to manage the pollution with uranium in soil. Various concentrations of uranium (0.0, 50,100, 200, 500 and 1000mg.l⁻¹) in the form of uranyl nitrate were added to MS medium that used for the initiation and growth of Acacia callus in the presence of 0.5 mg/l of each NAA and BA. For comparison, two species of Acacia were used in this study, *A. albida* and *A. nilotica*. Fresh and dry weight of callus was determined along three growth periods (30, 60 and 90 days). levels of uranium in callus cells were determined using TXRF analysis. The Results showed that fresh and dry weight of callus increased with the increase of uranium concentrations except that grown on MS with 1000 mg.l⁻¹ of uranium in which inhibition in both fresh and dry weight of callus after three months of growth was recorded .Analysis of the cellular content of uranium using TXRF revealed that the maximum level obtained when callus grown on MS with 500mg.l⁻¹ .

)
MS / (1000, 500 ,200 ,100 ,50 , 0.0) (NAA BA / 0.5
Acacia albida .
Acacia nilotica
.TXRF 90 60 30

TXRF
/ 500 MS

INTRODUCTION

Metal contamination found within food grown in Iraq exceeds current WHO (World Health Organization) levels in some of the regions and there is a serious concern about the presence of depleted uranium in Iraq. The Iraqi environment, in some regions, is heavily polluted by various toxic metals and radioactive pollution especially in the soil, water and air. So, any mechanism for reducing the environmental exposure to depleted uranium will greatly reduce the risk of human. Plants were suggested to be used in removal and stabilization of metal contaminants (Belimov, *et al.*, 2003; Meagher, 2000). Progress has been made in recent years by using plants and other organisms for the remediation of environmental contaminants. These technologies depend on different living organisms like plants, green algae, fungi, bacteria etc. (McGrath *et al.*, 1993). It was showed that plants might be used to remove metals from polluted soils (Cunningham *et al.*, 1997; Sas-Nowosielskaa, 2004).

Phytoremediation is the major part of bioremediation, a promising technology using plants to remove contaminants as heavy metals and radioactive elements from the environment rendering them harmless; it is actually a generic term for several ways in which plants can be used to clean up contaminated soils and water. The mechanisms of phytoremediation include enhanced rhizosphere biodegradation, phyto-extraction (also called phyto-accumulation), phyto-degradation, and phyto-stabilization and finally defined as the use of living green plants for cleaning up sites from organic and inorganic pollutant (Sas-Nowosielskaa, 2004). Uranium translocation among tissues of Indian mustard *Brassica juncea* plants is in the relation of roots > shoots > leaves, and Indian mustard is recommended as a potential species for phytoextraction for U-contaminated soil due to its high U accumulation of above ground biomass (2200 µg per plant) (Chang *et al.*, 2005).

Accumulation of Uranium in plant tissues was studied in the shoots and roots of corn—maize (*Zea mays*), grown on two types of soils, pseudogley and chernozem, together with its phytotoxic effect on plant growth and development. The soils were contaminated with different rates (10 to 1000 mg U(VI) kg⁻¹) of uranyl nitrate (UO₂(NO₃)₂·6H₂O), they found a linear dependence between the content of uranium in soil and in plant tissues (Stojanović *et al.*, 2010). Sunflower was shown to be very effective in recovering U from U-contaminated water. Uranium accumulated mainly in the roots, with concentrations 5,000 to 10,000 times greater than that in the water (Olowoyoa *et al.*, 2012; Huang *et al.*, 1998). Phytoremediation of uranium (U) contaminated soil has been hampered by a lack of information relating U speciation to plant uptake.

This study was carried out to understand the uptake of uranium by callus cells derived from two species of Acacia, *A. albida* and *A. nilotica* that's to make decision for choosing Acacia trees to be used in phytoremediation programs in the contaminated sites in Iraq. It is important to mention that this study is part of a project that uses the field experiments and

callus cultures of these trees. Acacia trees which belong to the family *Fabaceae* (*Leguminosae*), are one of the largest families of flowering plants, subfamily *Mimosoideae* mostly trees or shrubs numbering 630 genera and 18,000 species, there are approximately 2379 species in the *Acacia* genus, distributed from Africa around the Indian Ocean to tropical Asia and Australia and in the tropical Americas.

Acacia albida, Botanical Name: *Faidherbia albida*, is drought resistant and native in Africa and the Middle East and Arabia, the tree is widespread in semiarid areas of Africa. Over 20C. was usually found on a wide range of soil types and within varying climates and habitats it prefers semiarid, alluvial, revering zones and depressions with water present below the surface (Van Wyk, and Van Wyk 1997).

Acacia nilotica, very naturally widespread species in the drier areas of Africa from Senegal to Egypt and down to South Africa and in Asia from Arabia eastwards to India, Burma and Sri Lanka. It has also been cultivated elsewhere, including Australia, Cape Verde islands, Indonesia, Iran, Iraq, Nepal, Vietnam, and the West Indies. It is divided into nine subspecies. Usually 2.5-15 m tall, but reaching 25 m or more in the riverine subspecies, with root system deep and extensive in dry sites, the taproot developing first and then the laterals, which become compact and massive (Fagg and Greaves, 1990).

MATERIALS AND METHODS

Seeds of *A.albida* and *A. nilotica* were imported from -5 Pakistan (stored at 3-5⁰ C), by the Environment Center of Wales /Bangor University/UK, in which all the experiments of this study were carried out. The seeds were surface sterilized using ethanol 95% for two minutes. then 2% sodium hypochlorite (Sigma) for 12 minutes. followed by five separate rinses with sterile distilled water 2 minutes each. After then, the explants were transferred on sterilized filter paper. Sterilized seeds were cultured on Arnon and Hoagland medium (Arnon and Hoagland, 1944) for germination. Sterilized seedlings used as a source for explants were shown in (Fig.1).



Fig. 1 : Sterilized seedlings of *A.albida* that used as a source for the explants.

Source of explants:

Explants used for the initiation of callus were taken from a stem (explants used were both with node and without node, they are used for the initiation of callus), root, leaf, and cotyledons of 25-30 days seedlings. The cultures were kept on using growth cabinet supplemented with a 16 h light and 8 h dark, 25- 30°C day-night regime with 70% humidity. jars as shown in figure-1- or 9 cm I.D. Petri dishes were used in this study depending on the experiment. All these explants in addition to the callus were used for treatment with uranium concentrations as well.

Callus initiation & growth:

To initiate callus sterilized seedlings of 25-30 days old were excised into 1-1.5 cm explants for the stem and root, 0.5 cm² for the leaves and cotyledons, these explants were cultured on MS (Murashige and Skoog, 1962) medium, solidified with 7g/l of agar and addition of 35 g/l sucrose. Plant growth regulators used were 0.5 mg/l of each NAA and BA respectively. Callus was recultured on fresh medium (as mentioned above) every 30 days for maintenance of growth.

Determination of callus fresh and dry weight:

Callus derived from stem explants were cultured on MS medium as mentioned above but with the addition of various concentrations of uranium(as Uranyl Nitrate $UO_2(NO_3)_2$) (50,100,200,500 and 1000) mg/l ,then let to grow for 90 days for albida ,but for 60 days in case of nilotica for the reasons that this species was more sensitive for temperature and needs more time for callus initiation. In each 30 days period, fresh and dry weight was determined.

TXRF study:

Callus grown on MS media with the different conc. (50, 100, 200, 500, and 1000mg/l) of Uranium were analyzed by TXRF (Total Reflection X-ray Fluorescence Spectrometer) (Bruker,S2 PICOFOX , USA).

For that callus dried at 80 °C for 24 hours, grinded by ball mill and sieved to < 25 µm to remove any large particle. Between 20-30 mg of each sample was diluted with 1% Triton in aliquot.10 µl of arsenic was added as standard. 5 µl of the homogenised sample was applied directly into a polished carrier made of quartz or acrylic glass, and dried on a hot plate (80 °C). The discs were then placed in the TXRF for the analysis of uranium and other elements in plants by a quantitative analysis.

RESULTS

Response of seedling parts to initiate Callus:

The highest response for callus initiation was observed in seedling stem segments that showed 83% response as compared to 26% for the root explants and 13% for the cotyledons explants, whereas leaf explants respond with a very low rate 2% (Fig. 2), these results were expected ,if we know that stem contains the high ratio of parenchymatous cells as compared with root, cotyledons and leaves, which considered to be the target cells that got the signal and initiate callus. The results showed that callus formed from stem, root and cotyledons was friable yellowish (Fig. 3 and 4).

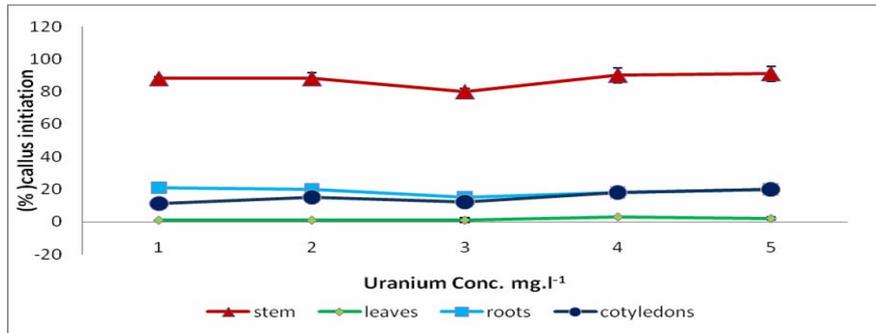


Fig. 2: Response (%) of different seedling explants sources to initiate callus using MS medium with the addition of 0.5 mg.l^{-1} of BA and NAA and different conc. of Uranium as Uranyl nitrate.

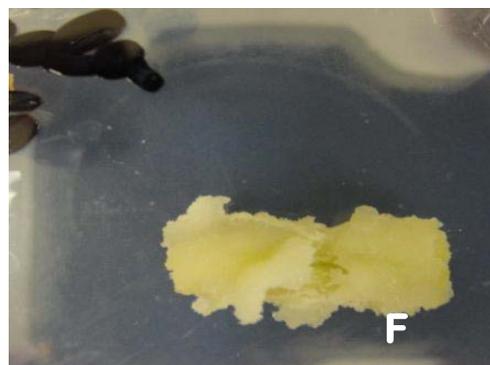
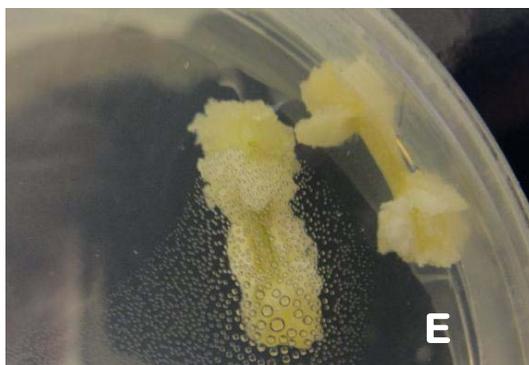


Fig. 3 : Response of seedling stem explants to initiate callus after 30 days of culture on MS medium supplemented with 0.5 mg.l^{-1} of each BA and NAA and different concentrations of Uranium: A-Control, B- 50, C- 100, D-200, E-500, F-1000 mg.l^{-1} .

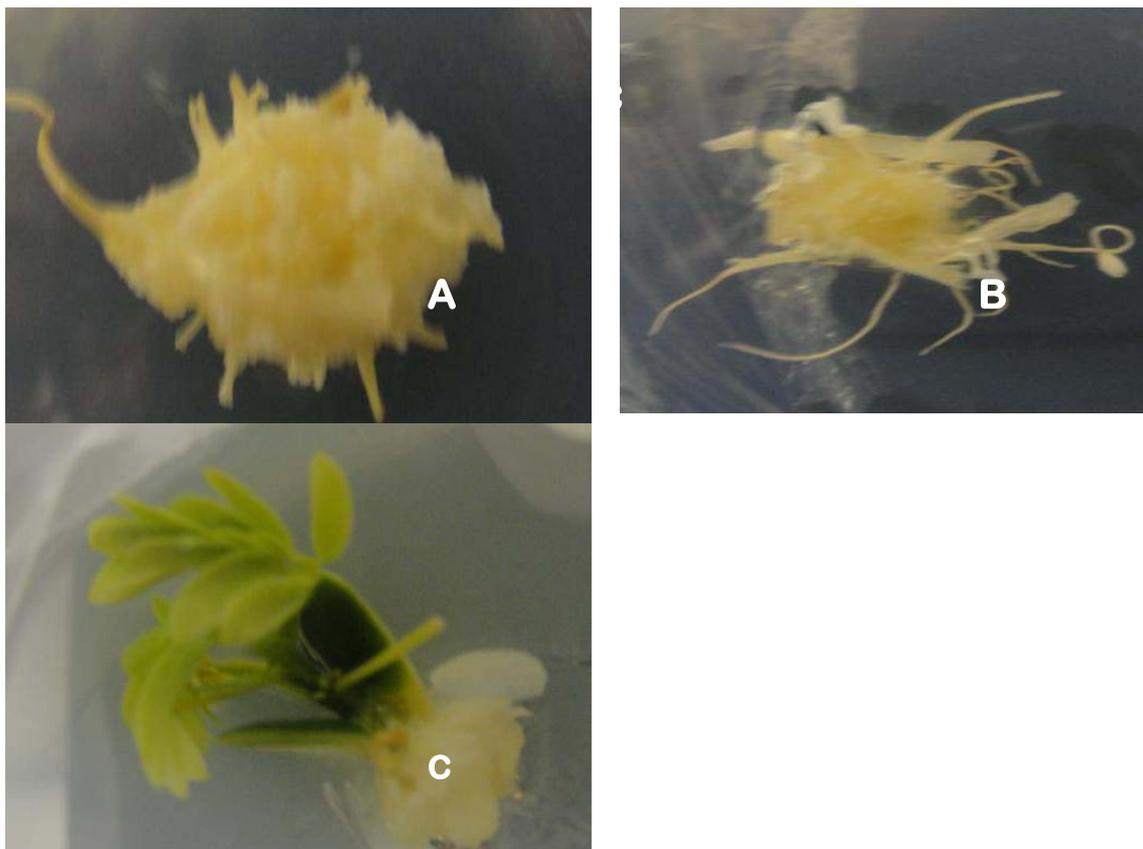


Fig. 4: Response of root and Cotyledon explants to initiate callus after 30 days of culture on MS medium supplemented with 0.5 mg.l⁻¹ of each BA and NAA and different concentrations of Uranium: A-root explants on the Control, B-root explants on 500 mg.l⁻¹, C- cotyledons explants on 500 mg.l⁻¹.

Callus Fresh Weight

A. *Acacia albida*

Fig. 5 shows that callus fresh weight fluctuated with the concentration of uranium in the medium. At the first stage of growth (30 days), the results showed that callus fresh weight in the treatment of 100 and 200 mg.l⁻¹ uranium was 1.47 and 1.42 g, which is very close to that (1.46 g) for the control, whereas with all other treatments of uranium (50, 500, 1000 mg.l⁻¹) callus fresh weight was less. The lowest fresh weight (0.88 g) was for the callus treated with 1000 mg.l⁻¹, this probably because a high concentration may have a negative effect on cell growth, Stojanović *et al.*, (2010) studied the accumulation of Uranium in plant tissue shoots and roots of corn—maize (*Zea mays*), grown on two types of soils, pseudogley and chernozem, together with its phytotoxic effect on plant growth and development, they reported that the soils were contaminated with different rates (10 to 1000 mg U(VI) kg⁻¹) of uranyl nitrate.

In the second and third stage of growth (60 and 90 days), callus fresh weight gave the highest values (2.02 and 2.32 g) in the treatment of 500 mg.l⁻¹ while in other treatments, callus fresh weight was less, this is probably due to the role of nitrate within the uranium that enhances callus growth, that's because we used uranium in the form of uranyl nitrate.

The statistical analysis showed that it is highly significant $P \leq 0.01$. The callus seems to be friable with yellowish white color.

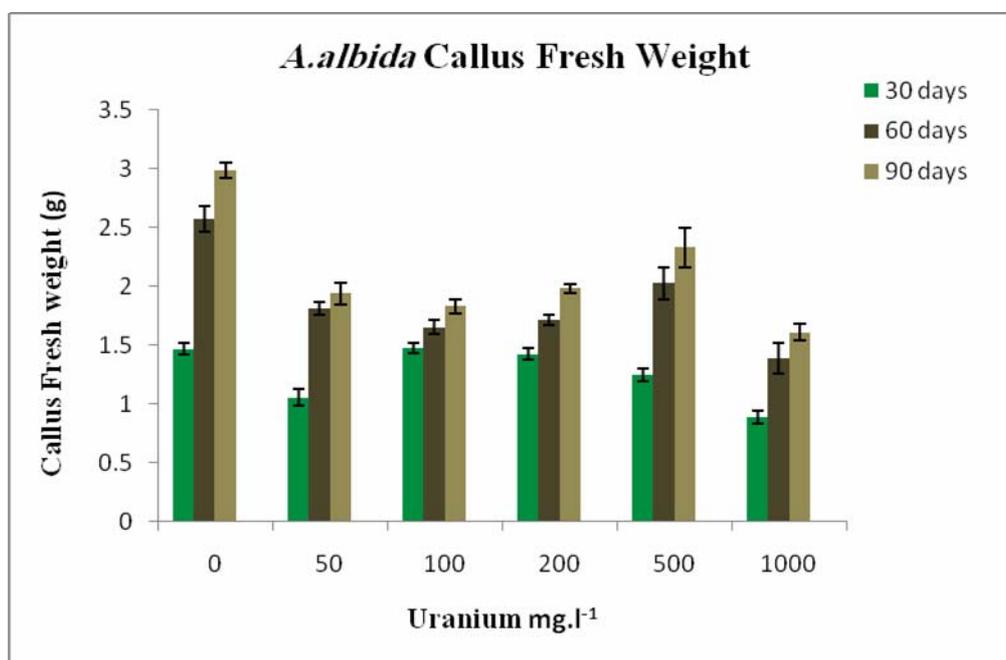


Fig. 5: *A.albida* Callus fresh weight after 30,60 and 90 days of growth on MS medium with 0.5 mg.l⁻¹ of BA and NAA ,and addition of different conc. of Uranium.

B - *Acacia nilotica*

Callus fresh weight of *A.nilotica* showed to be highly significant $P \leq 0.01$ for the concentration and age but for the relation between conc. and age the statistical analysis showed to be non-significant. The fresh weight of callus (Fig. 6) varied with the variation of uranium concentrations in the medium, it seems that the stimulation for callus initiation and growth is less than that in *A.albida*. callus fresh weight in the control treatment reached to 0.93,1.37 g after 30 and 60 days of growth compared with 1.46 and 2.57 g for the control treatment of *A.albida* (Fig. 5), this is probably due to that explants from *A. nilotica* did not respond in the same manner as other species in tissue culture systems, or it is due to the less active seedlings of *A. nilotica* compared with *A.albida* but when treated with uranium, the results showed that callus growth increased with the increase of uranium concentrations (50-1000 mg.l⁻¹). This result leads to the fact that *A. nilotica* can accumulate higher conc. of uranium, as compared with *A. albida* in which the callus fresh weight decreased when treated with 1000 mg.l⁻¹ as mentioned above. The callus seems to be more compact in texture than that of *albida* (Fig. 7). In general, it is clear that the growth of *A. nilotica* callus is not active as with *A. albida*, for that we depended on *A. albida* in all the following experiments related to the uptake of uranium and its effect on fresh and dry weight.

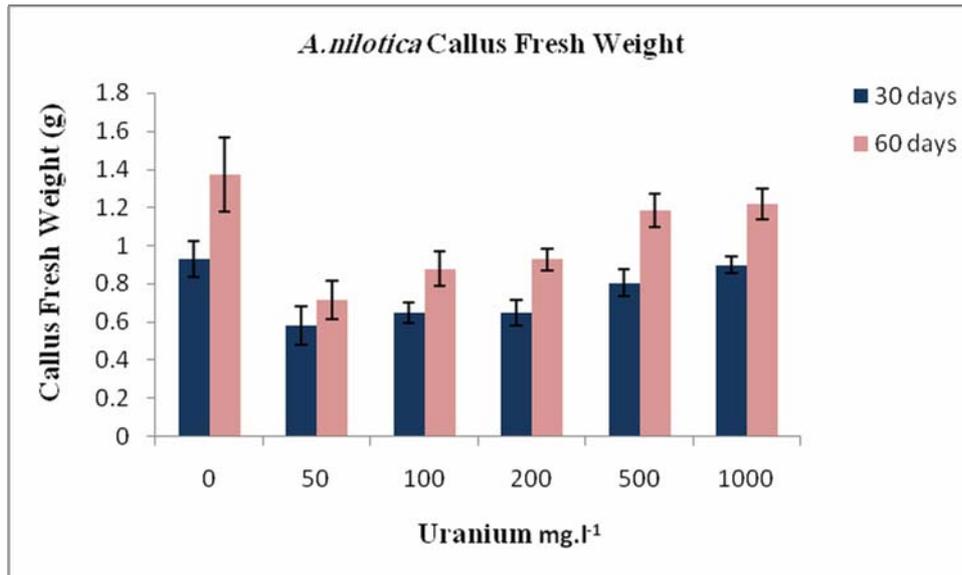


Fig. 6: *A. nilotica* Callus fresh weight after 30,60 days of growth on MS medium with 0.5 mg.l⁻¹ of BA and NAA ,and addition of different conc. of Uranium .

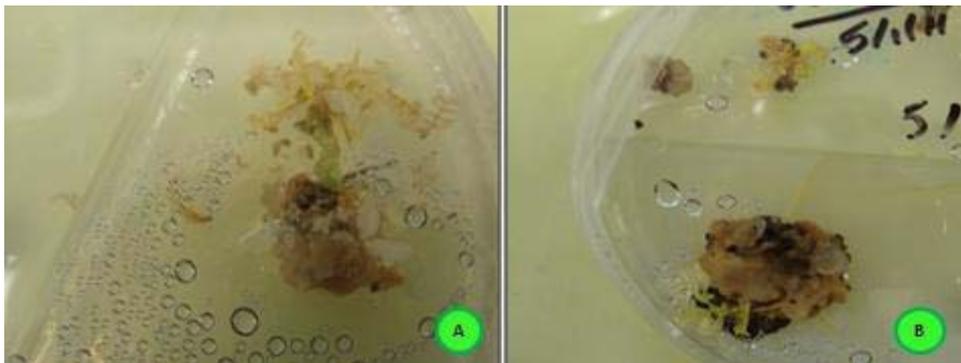


Fig. 7 : *A. nilotica* callus grown for 50 days on MS medium with A-500, B- 1000 mg.l⁻¹ of uranyl nitrate.

Dry Weight

A- *A. albida*

The highest value of callus dry weight is shown to be 0.263 and 0.267 g for the treatment with 200 and 500 mg.l⁻¹ respectively, while the most reduction in dry weight was recorded with the treatment of 50 and 1000 mg.l⁻¹, this is in the case of the first 30 days of growth (Fig. 8). but for the 60 and 90 days of growth the results showed that the most reduction in dry weight happened with the treatment of 100 and 200 mg.l⁻¹ of uranium in which the dry weight reached to (0.312 and 0.311g) after 60 days and 0.356,0.34 g for 90 days. It is clear from the results in (Fig. 8) that after 90 days of growth dry weight for the treatments of 500 and 1000 mg.l⁻¹ reached the highest values of 0.421 and 0.390 which are near to that of the control treatment (0.465g). The statistical analysis showed that it is highly significant $P \leq 0.01$ for the age and uranium concentrations, but it is non-significant for the relation between age and concentrations.

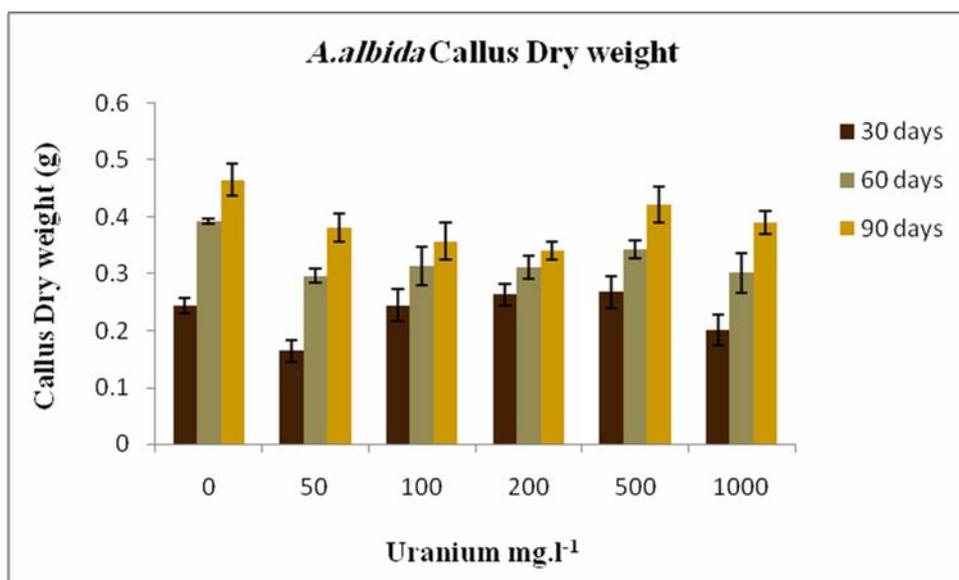


Fig. 8: Callus dry weight of *A.albida* after 30,60 and 90 days of growth on MS medium with addition of 0.5 mg.l⁻¹ of each BA and NAA and concentrations of Uranium.

B- *A.nilotica*

Callus dry weight of *A.nilotica* showed to be highly significant $P \leq 0.01$ for the conc. and the age but it is non significant for the relation between them. The results showed that the dry weight reduced as compared with that of *A.albida*. In the two stages of growth 30 and 60 days the dry weight showed a positive correlation with the uranium conc. used (Fig. 9). Also it is similar to the mode of increase in fresh weight (shown in Fig. 6). All the values of dry weight in all the treatments showed to be less than the control treatment, except that for the 1000 mg.l⁻¹ that reached to levels of 0.124 and 0.153 g after 30 and 60 days which are close to those in the control treatment (0.148 and 0.21 g) in the same stages.

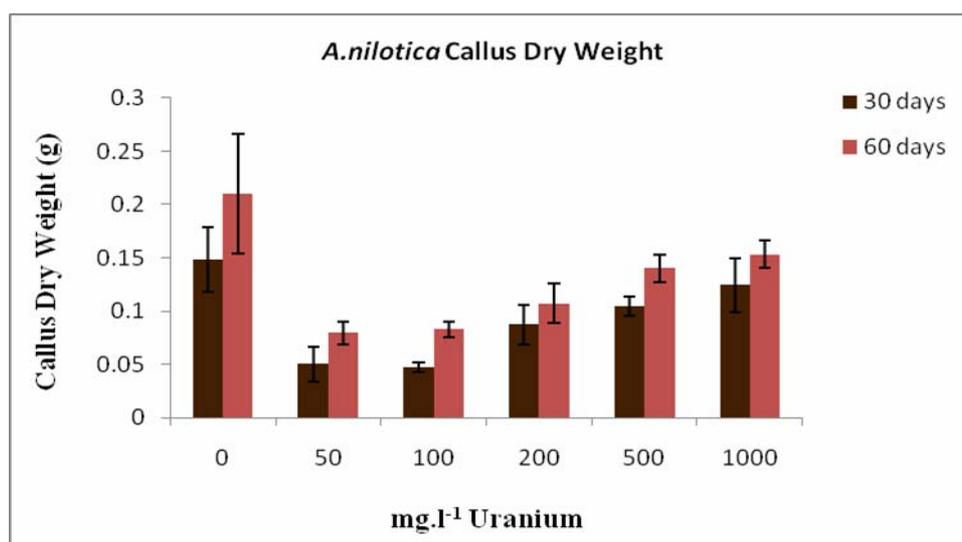


Fig. 9. : Callus dry weight of *A.nilotica* after 30 and 60 days of growth on MS medium with addition of 0.5 mg.l⁻¹ of each BA and NAA and concentrations of Uranium.

TXRF analysis:

Results of TXRF analysis (Fig. 10) showed that in case of using uranium in the form of Uranyl nitrate, the uptake of uranium increased with the increase of conc. 50 -500 mg.l⁻¹, after that there is a decrease with the treatment of 1000 mg.l⁻¹, but it is still with higher value than those for the treatments of 50,100 and 200 mg.l⁻¹. The Statistical analysis showed that it is HS $P \leq 0.01$ in comparison with the above results of fresh weight. Results showed that the fresh weight also decreased in the treatment of 1000 mg.l⁻¹, this is probably due to that *A.albida* callus could accumulate uranium and make use of the nitrate (Uranyl nitrate) to enhance growth, but with 1000 mg.l⁻¹ it is clear that the growth of callus affected by both uranium and nitrate high conc, especially if we keep in mind the conc, of nitrate already exist in MS medium.

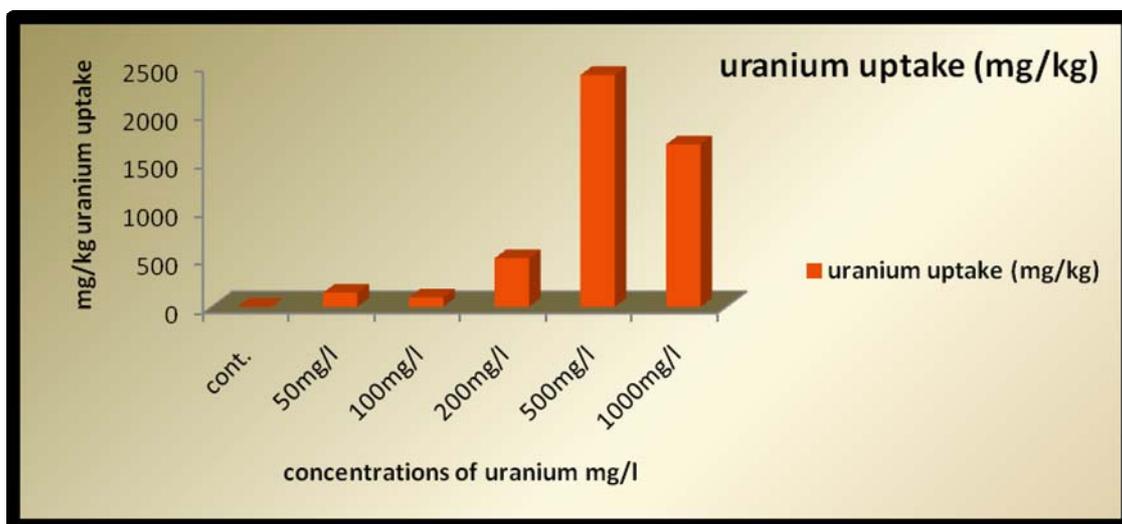


Fig. 10: TXRF analysis of uranium uptake by *A. albida* callus after 60 days of growth on MS medium supplemented with 0.5 mg.l⁻¹ of each NAA and BA and different concentrations of uranium.

DISCUSSION

Plant tissues grown in vitro provide an ideal research tool for the study of a wide range of aspects of plant science. It is also being increasingly adopted for the commercial propagation of plants (George *et al.*, 2008; Karuppusamy, 2009; Rai, 2007).

Tissue culture techniques offer not only an excellent opportunity to study the factors that elicit the totipotentiality of cells but also allow the investigation of factors controlling cytological and histological differentiation (Bhojwani and Razdan, 1996).

The results of this project revealed that stem explants were the best source to initiate callus, whereas leaves, root and cotyledons explants exhibit callus initiation but with a longer time and less growth average, these results may be due to that stem contains the high ratio of parenchymatous cells as compared with root, cotyledons and leaves, which were considered to be the target cells that got the signal and initiate callus (Razdan, 2003). For that reason, seedling stem explants were used in this study in all the experiments of callus initiation. Many studies referred to considered stem explants as the best source for callus initiation. In the system of tissue culture, callus induction depends on the parenchyma cells

that possess totipotency ability; however, sterile seedlings and not woody stem tissue tend to facilitate ease of callus initiation (George *et al.*, 2008).

One of the successful reports that refers to callus formation and plantlets are developed from five *Acacia* species, *A. senegal*, *A. albida*, *A. nilotica*, *A. koa* and *A. ligulata*. These reports also include callus raised through seedling shoot tip cultures of *Acacia koa*, cotyledonary buds of *A. albida*, stem cultures of *A. senegal*, shoot cultures from mature plants of *A. ligulata* and regeneration of nodes and internodes of field grown young twigs of *A. nilotica* (Mittal *et al.*, 1989). Another study in 1995 (Rout *et al.*, 1995) reported that callus also derived from immature cotyledons of *Acacia catechu* Willd. Cotyledonary segments expanded on all media 8-10 days after inoculation. Callus formed on the entire surface and developed into a mass of callus after 16-18 days of culture depending on the auxin and cytokinin concentration in the culture media. It was found that media supplemented by either NAA or 2,4-D along with kinetin or BA stimulated rapid proliferation of callus. The largest callus formation was obtained with WPM (woody plant medium) supplemented with kinetin, NAA and 3% sucrose and callus formed faster in the dark than in the 16-h photoperiod. They noticed that the explants cultured on WPM containing 2,4-D produced friable yellowish-brown callus, whereas callus formed in presence of NAA was more compact and deep yellowish (Rout *et al.*, 2008).

Results for the uptake of uranium by callus cells showed that uranium uptake increases with the increase of its concentration until reaching 500 mg l⁻¹. After that, uptake decreased (when addition of 1000 mg l⁻¹ to the medium). Also callus growth was not affected by uranium concentration during the first two months of growth, but from the third month and thereafter callus growth slightly decreased and browning appeared on callus cultures. Although reculture of callus after each 30 days helped to recover its activity, its growth never recovered to that of the control treatment, and this means that a level of uranium was being accumulated in the cells and affecting callus viability. These results were supported by the TXRF analysis which showed the same model for uranium uptake. This led to the conclusion that acacia callus could accumulate uranium. To date about 400 plants that hyperaccumulate metals have been reported. The dominating hyperaccumulating families are *Asteraceae*, *Brassicaceae*, *Caryophyllaceae*, *Cyperaceae*, *Cunouniaceae*, *Fabaceae*, *Flacourtiaceae*, *Lamiaceae*, *Poaceae*, *Violaceae*, and *Euphobiaceae* (Prasad and Freitas, 2003).

Growth in vitro is highly dependent on the interaction between naturally occurring endogenous growth substances and an analogous growth regulator added to the medium. The origin of the explants, the type of culture, and other medium constituents are also important factors affecting callus initiation and growth. Callus composed of cell aggregates of parenchyma cells obtains nutrients directly from the medium, and in this it differs from the organized tissues in the whole plants (Hartmann *et al.*, 2002). The suggestion here that callus cells are sensitive indicators for toxic elements (like uranium in our study). The similar differential sensitive form between the two *Acacia* species grown both as callus and whole plants, suggest that callus could be used to assess plants for phytoremediation programs.

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