







AN EFFICIENT PROTOCOL FOR *IN VITRO* REGENERATION OF
PEANUT (*Arachis hypogaea* L.) CULTIVAR L14

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How to cite: HOA, P.T.B., et al. An efficient protocol for *in vitro* regeneration of peanut (*Arachis hypogaea* L.) cultivar L14. *Bioscience Journal*. 2021, **37**, e37019. <https://doi.org/10.14393/BJ-v37n0a2021-56949>

Abstract

The present work aims to establish an efficient protocol for *in vitro* regeneration of peanut (*Arachis hypogaea*) cultivar L14. The study showed that de-embryonated cotyledon was a suitable explant for shoot multiplication on MS medium containing 4 mg/L BAP. The highest number of shoots per explant obtained after 4 weeks of culture was up to 6.8 shoots. Shoots *in vitro* were able to produce a large number of approximately 11 roots on MS medium supplemented with 0.5 mg/L NAA. These results will be very useful in establishing an *in vitro* regeneration protocol for peanut cultivar L14 during gene transfer in the next studies to improve their disease resistance.

Keywords: *Arachis hypogaea*. *In Vitro* Propagation. Rooting. Shoot Multiplication. Shoot Regeneration.

1. Introduction

Arachis hypogaea (peanut, groundnut), an annual oilseed crop of the Fabaceae family, is native to South America but is currently grown in diverse environments around the world (Sharma and Bhatnagar-Mathur 2006). Peanut is also known as an important industrial and oilseed crop in many of the warmer regions of the world. Peanut seed contains about 50% oil (Cheng et al. 1992); a rich source of protein (22-30% dry weight), many minerals (P, Ca, Mg and K), and vitamins (Savage and Keenan 1994). However, pests and diseases are two of the causes leading to poor peanut productivity (Nageshwara-Rao and Nigam 2001). Therefore, the transfer of genes for resistance to pests and diseases into peanuts is considered a promising solution to increase field productivity (Dang et al. 2019; Vasavirama and Kirti 2012).

To transfer genes into plants, completing an efficient *in vitro* regeneration system for them is a very important first step. In literature, numerous studies on *in vitro* cultures of peanut were reported previously, such as micropropagation from mature cotyledons (Venkatachalam et al. 1999; Maina et al. 2010), nodals (Al-Joboury et al. 2012), de-embryonated cotyledon (Radhakrishnan et al. 2000; Pratap et al. 2018) and cotyledonary nodes (Verma et al. 2011, 2014; Limbua et al. 2019); or through organogenesis (Pestana et al. 1999), somatic embryogenesis and callus culture (Iqbal et al. 2011). However, studies have also shown that the response of peanut cultivars to the culture medium is very different. It is almost difficult to use a nutrient medium for this peanut cultivar to culture the others if a high regeneration efficiency is desired.

Peanut cultivar L14 (Spanish group) has a high yield from 4-4.5 metric tons/ha and is suitable for many peanut growing areas in Vietnam. Plants have good agronomic characteristics such as vertical stem (30-50 cm high), neat canopy, good anti-falling, and dark green leaves. The growing period from 120-125

days for a spring crop, 95-100 days for an autumn-winter crop. 100-seed weight from 58-60 g, kernel/fruit ratio from 73-75%. This peanut cultivar is highly resistant to some leaf spot diseases such as brown spots, black spots, rust, and bacterial wilt (Suu et al. 2017), but is poorly resistant to stem rot caused by white mold (*Sclerotium rolfsii*) (Cuong et al. 2018). Therefore, in the present study, we investigated the *in vitro* regeneration capacity of different types of explants from peanut cultivar L14 to serve genetic transformation in the next study to improve the white mold disease resistance of peanuts.

2. Material and Methods

Studies were performed in peanut (*Arachis hypogaea* L.) cultivar L14 which was provided by Field Crops Research Institute, Vietnam Academy of Agricultural Sciences. Explants were used for *in vitro* cultures including hypocotyl, epicotyl, shoot-tip, and cotyledon node from germinated seeds, embryonated cotyledon, and *de*-embryonated cotyledon. Cultures of shoot regeneration and multiplication, and root induction were maintained at 25±2°C at the cool white, the fluorescent light intensity of 27 μmol/m²/s with 12 h daylight. *In vitro* growth in all experiments was evaluated at week 4 of culture.

Healthy peanut seeds were carefully rinsed under running water, then surface sterilized with 65% sodium hypochlorite solution for 10 minutes. Finally, the seeds are rinsed four times with sterile distilled water. Sterile seeds were germinated on basal MS (Murashige and Skoog 1962) medium containing 3% sucrose and 0.8% agar, pH of 5.8. Explants (5 days old) were cultured on MS medium containing 3% sucrose and 0.8% agar, supplemented with various concentrations of benzylaminopurine (BAP) from 1-10 mg/L and naphthaleneacetic acid (NAA) from 0.1-0.5 mg/L to induce shoot regeneration. *In vitro* individual shoots were then transferred to MS medium containing 3% sucrose and 0.8% agar, supplemented with BAP from 1-5 mg/L for shoot multiplication. Shoots (approx. 3 cm length) were isolated from *in vitro* shoot clusters and subcultured on MS medium containing 3% sucrose and 0.8% agar, supplemented with NAA or indolebutyric acid (IBA) from 0.1-0.5 mg/L for rooting.

3. Results and Discussion

The present study showed that more than 81% of sterile peanuts cultivar L14 have germinated on MS medium after 3 days of incubation (Figure 1A) and by day 7 have grown into *in vitro* whole plant. Explants including shoot-tip, hypocotyl, epicotyl, and cotyledon nodes were isolated from seedlings to investigate shoot regeneration on different culture media (Data not shown). The observation found that MS medium containing 4 mg/L BAP and 0.1 mg/L NAA is the most suitable. The number of regenerated shoots from epicotyl (2.3) was higher than the remaining types of explants (1-1.8), however, regenerating explants were only about 50%.

The highest shoot regeneration efficiency was found in the shoot-tips, shoot regenerating explants of 95% with 1.6 shoots per explant (Table 1).

Table 1. Shoot regeneration from various types of explants on MS medium containing 4 mg/L BAP and 0.1 mg/L NAA.

Explants	Regenerating explants (%)	No. of shoots per explant	Shoot length (cm)
Shoot-tip	95	1.6 ^b	3.1 ^b
Epicotyl	50	2.3 ^a	2.9 ^b
Hypocotyl	40	1.8 ^b	2.6 ^b
Cotyledon node	100	1.0 ^b	6.6 ^a

Different letters in a column indicate significantly different means (Duncan's test, $p < 0.05$).

As it is well known, cytokinins and auxins such as benzyladenine (BA), BAP, 2-isopentenyladenine (2-iP), thidiazuron (TDZ), and NAA are commonly used for plant micropropagation. Cytokinins and auxins play a crucial role as promoters of cell division and act in the induction and development of meristematic centers leading to the formation of organs (Peeters et al. 1991). Shoot-tip (0.1-1 mm high) is the terminal portion of a shoot comprising the apical meristem (0.05-0.1 mm) together with primordial and developing leaves and adjacent stem tissue (Torres 1989). Hence, the majority of shoot-tip culture is essentially apical meristem

cultures. In our thinking, explants taken from actively growing parts of plants such as apical meristem are very suitable for shoot multiplication. And a combination of BAP and NAA in an appropriate ratio and concentration will facilitate multiple axillary shoot initiation and elongation on the main explant (Rasool et al. 2009).

In vitro shoots from regenerations were subcultured on MS medium supplemented with BAP from 1-5 mg/L. The highest shoot number obtained at 4 mg/L BAP with 3.8 shoots per explant, regenerating explants of about 40% was relatively high compared to other concentrations of BAP (Table 2 and Figure 1B).

Table 2. Effect of BAP on shoot multiplication of individual shoots from regeneration cultures.

BAP (mg/L)	Regenerating explants (%)	No. of shoots per explant	Shoot length (cm)
1	18.8	2.6 ^{bc}	2.4 ^{ab}
2	21.9	2.9 ^{ab}	2.6 ^{ab}
3	31.3	3.1 ^{ab}	2.7 ^{ab}
4	40.6	3.8 ^a	3.0 ^a
5	43.8	3.0 ^{ab}	2.9 ^{ab}
Control	12.5	1.9 ^c	2.3 ^b

Different letters in a column indicate significantly different means (Duncan's test, $p < 0.05$). Control is medium without BAP.

Some previous reports showed that *in vitro* shoot proliferation of peanut has been found at higher BAP concentrations, e.g. MS medium containing 33 μ M (over 7.4 mg/L) benzyladenine (BA) in combination with 5.3 μ M NAA and 23.54 μ M AgNO₃ recorded maximum average shoot number per regenerating explant (shoot-tip) was 6.3 (Ozudogru et al. 2005). Palanivel et al (2014) obtained the highest number of shoots (over 17 shoots) in peanut cultivar ICGV00351 (Tamil Nadu, India) on a medium containing 25 mg/L BAP and 0.5 mg/L indoleacetic acid (IAA). In another study, different explants such as zygotic embryos, plumular apices, and embryonic axes of peanut cultivar NC-7 were pretreated for 15 days on MS medium supplemented with 25 mg/L BA. Subsequent culture counted the highest shoot counts per explant ranging from 2.67 to 4.43 (zygotic embryos), 2.33 to 4.11 (plumular apices) and 1.87 to 3.33 (embryonic axes) on MS medium containing BA concentrations ranged from 0.25 to 3 mg/L after 8 weeks of observation (Özkan and Aasim 2019).

To improve shoot multiplication rate, embryonated cotyledons and de-embryonated cotyledons were cultured on MS medium supplemented with different concentrations of BAP from 1-5 mg/L for investigation of shoot proliferation. Data from table 3 shows that regeneration of de-embryonated cotyledons peaked with 6.8 shoots per explant, and 1 shoot for embryonated cotyledons in medium with 4 mg/L BAP (Figure 1C). Other tested concentrations of BAP showed lower efficiency for shoot regeneration (Data not shown).

Table 3. Shoot regeneration from embryonic cotyledons on MS medium containing 4 mg/L BAP.

Explants	Regenerating explants (%)	No. of shoots per explant	Shoot length (cm)
De-embryonated cotyledon	23.3	6.8 ^a	4.1 ^b
Embryonated cotyledon	86.7	1.0 ^b	5.9 ^a

Different letters in a column indicate significantly different means (Duncan's test, $p < 0.05$).

It has been reported that de-embryonated cotyledons of oilseed crops (e.g. peanut) are highly regenerating without callus intervention, so they were often used as a type of potential explant for shoot multiplication (Tiwari and Tuli 2008; Radhakrishnan et al. 2000). A study of Tiwari and Tuli (2008) in four peanut cultivars of India (JL-24, TMV-2, TAG-24, and Dh-3-30) indicated that adventitious shoot primordia could be induced from de-embryonated cotyledon explants were cultured in a medium containing 5 mg/L BAP and 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) for 4 weeks, or in medium containing only BAP at 20 mg/L for the first 2 weeks, followed by 15 mg/L BAP for the next 2 weeks. Therefore, the results in the present study have once again contributed to reinforcing the above.

However, Radhakrishnan et al (2000) only obtained 57.9% of multiple shoot formation from de-embryonated cotyledons in peanut cultivar J11 in the medium containing 15 mg/L BAP. Whereas, embryo

axes and mature whole seeds can achieve to the highest of 100% and 86% in the medium supplemented with 30 and above and 50 mg/L BAP, respectively. In a similar study subjected to multiple shoot induction in two peanut cultivars, VRI-2 and VRI-3 from Tamil Nadu (India), Palanivel and Jayabalan (2002) found that (whole) embryonated cotyledon produced more well-developed shoots in both cultivars. The (whole) de-embryonated cotyledon and sectional de-embryonated cotyledon explants showed poor response. Among the different concentrations of BAP combined with 0.5 mg/L NAA, 25 mg/L BAP was considered to be the best in terms of the highest mean number of shoots and the shortest shoot induction time. In general, shoot induction of de-embryonated cotyledons, as well as other types of explants, was very different depending on the peanut cultivar. The number of shoots produced per explant varied with the concentration of plant growth regulators in the medium.

In vitro peanut shoots were transferred onto MS medium containing NAA and IBA for root induction. The data in table 4 indicate that all tested concentrations of two of these plant growth regulators stimulated rooting. However, NAA showed higher efficacy than IBA, the highest number of roots was 10.9 at a concentration of 0.5 mg/L with a regeneration rate of 100% and roots were formed earlier, only after 9-12 days of culture (Figure 1D). Whereas on the IBA-containing medium, the *in vitro* shoots had a smaller number of roots, and roots formed later, from day 15 to day 18 (Data not shown).

Table 4. Effect of NAA and IBA on rooting of *in vitro* shoots.

Plant growth regulator (mg/L)	Shoots regenerating roots (%)	No. of roots per rooted shoot	Root length (cm)
NAA	0.1	75	4.3 ^b
	0.3	87.5	4.4 ^b
	0.5	100	10.9 ^a
IBA	0.1	75	3.4 ^b
	0.3	62.5	2.6 ^c
	0.5	50	1.9 ^c
Control	0.0	50	1.5 ^c
			4.9 ^{ab}

Different letters in a column indicate significantly different means (Duncan's test, $p < 0.05$).

The effects of auxin group of hormones (e.g. NAA, IBA, or IAA) on rooting and plant development have been discussed in several previous studies depending on the induction of various explants or species. However, in some cases, NAA stimulated root regeneration better than IBA. For example, Limbua et al (2019) also found that NAA is the suitable growth regulator for peanut cultivars CGV 12991, CG 7, and Red Valencia, and if using a concentration of 1 mg/L can obtain the highest number of plants with roots. Some previous studies in other plant species have also shown similar results, e.g. *in vitro* root regeneration in sugarcane (Jagadeesh et al. 2011) or in bromeliads (*Neoregelia concentrica*) (Martins et al. 2013).

In contrast, peanut cultivar ICGV-00351 only gave high root regeneration, up to 25 roots per explant which derived from (whole) embryonated cotyledon, when grown on medium with 5 mg/L IBA (Palanivel et al. 2014). The *in vitro* shoots of the *E. grandis* × *E. urophylla* hybrid had also a good response with IBA for rooting (Cid et al. 1999). Whereas the best *in vitro* rooting of the apple 'Jork 9' shoots was found in media containing IAA.

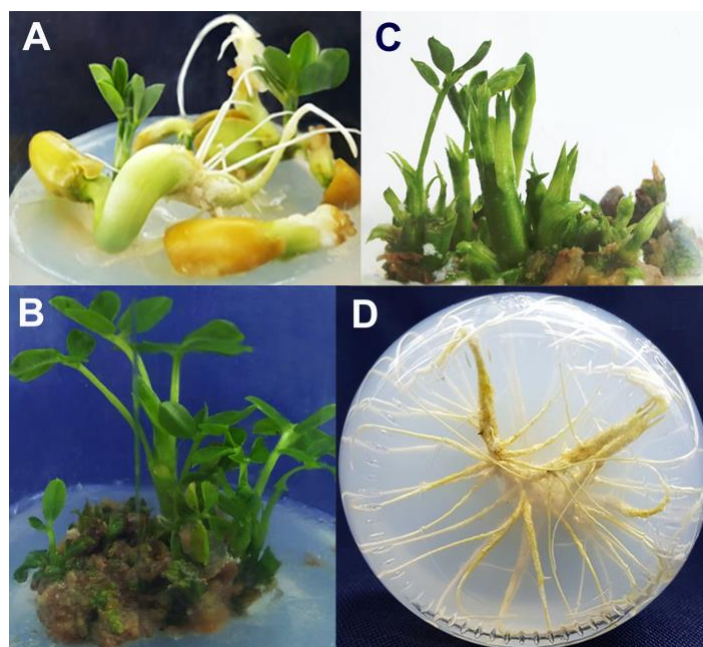


Figure 1. *In vitro* cultures of peanut. A – germination of seeds; B – shoot multiplication from individual shoots; C – shoot regeneration from de-embryonated cotyledons; D – rooting of individual shoots.

4. Conclusions

In conclusion, the current study shows that the de-embryonated cotyledon of peanut cultivar L14 is a type of suitable explant and that it may give high efficiency in shoot proliferation on a medium containing 4 mg/L BAP. Rooting of individual shoots can peak at a concentration of 0.5 mg/L NAA.

Authors' Contributions: HOA, P.T.B.: conception and design, acquisition of data, analysis and interpretation of data, and drafting the manuscript; TUE, N.H.: conception and design, acquisition of data, analysis and interpretation of data, and drafting the manuscript; TRANG, P.T.Q.: acquisition of data, analysis and interpretation of data, and drafting the manuscript; HANG, L.T.: acquisition of data, analysis and interpretation of data, and drafting the manuscript; TIEN, N.Q.D.: acquisition of data, and drafting the manuscript; LOC, N.H.: conception and design, analysis and interpretation of data, and drafting the manuscript. All authors have read and approved the final version of the manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

Ethics Approval: Not applicable.

Acknowledgments: This work was supported by the National Foundation for Science and Technology Development (NAFOSTED) - Vietnam, Finance Code 106.02-2017.346. The authors would also like to thank Hue University, Vietnam for supporting this research.

References

- AL-JOBOURY, K.H.R. *In vitro* propagation of groundnut (*Arachis hypogaea* L.). *Ibn Al-Haitham Journal for Pure and Applied Science*. 2012, **3**(2), 13-19.
- CHENG, M., HIS, D.C.H. and PHILLIPS, G.C. *In vitro* regeneration of Valencia-type peanut (*Arachis hypogaea* L.) from cultured petioles, epicotyl sections, and other seedling explants. *Peanut Science*. 1992, **19**(2), 82-87. <https://doi.org/10.3146/i0095-3679-19-2-5>
- CID, L.P.B., et al. Plant regeneration from seedling explants of *Eucalyptus grandis* x *E. urophylla*. *Plant Cell, Tissue and Organ Culture*. 1999, **56**(1), 17-23. <https://doi.org/10.1023/A:1006283816625>
- CUONG, L.N., NHUNG, N.T. and DIEM, N.T. Fungal inhibition and disease control of groundnut stem rot (*Sclerotium rolfsii*) by nanosilver liquid. *Hue University Journal of Science: Agriculture and Rural Development*. 2018, **127**(3A), 161-171.
- DANG, P.M., et al. Identification of expressed *R*-genes associated with leaf spot diseases in cultivated peanut. *Molecular Biology Reports*. 2019, **46**(1), 225-239. <https://doi.org/10.1007/s11033-018-4464-5>
- IQBAL, M.M., et al. *In vitro* micropropagation of peanut (*Arachis hypogaea*) through direct somatic embryogenesis and callus culture. *International Journal of Agriculture & Biology*. 2011, **13**(5), 811-814.
- JAGADEESH, B., et al. Amenability of the sugarcane variety 2005 T 16 to shoot tip culture. *Journal of Sugarcane Research*, 2011, **1**(2), 75-77.
- LIMBUA, P.G., NGUGI, M.P. and ODUOR, R.O. *In vitro* regeneration protocol of Kenyan adapted groundnut (*Arachis hypogaea* L.) genotypes using cotyledonary node explants. *Journal of Plant Biochemistry & Physiology*. 2019, **7**(1), 1000233. <https://doi.org/10.4172/2329-9029.1000233>

- MAINA, S.M., et al. Surface sterilant effect on the regeneration efficiency from cotyledon explants of groundnut (*Arachis hypogaea* L.) varieties adapted to eastern and Southern Africa. *African Journal of Biotechnology*. 2010, **9**(20), 2866-2871.
- MARTINS, J.P.R., et al. Effect of synthetic auxins on *in vitro* and *ex vitro* bromeliad rooting. *Pesquisa Agropecuária Tropical*. 2013, **43**(2), 138-146. <https://doi.org/10.1590/S1983-40632013000200009>
- MURASHIGE, T. and SKOOG, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*. 1962, **15**(3), 473-497. <https://doi.org/10.1111/j.1399-3054.1962.tb08052.x>
- NAGESHWARA-RAO, R.C. and NIGAM, S.N., 2001. Genetic options for drought management for groundnut. In: SAXENA, N.P., ed. *Management of agricultural drought: Agronomic and Genetic Options*, New Delhi: Oxford & IBH Publishing Co. Pvt. Ltd., pp. 123-141.
- OZUDOGRU, E.A., OZDEN-TOKATLI, Y. and AKCIN, A. Effect of silver nitrate on multiple shoot formation of Virginia-type peanut through shoot tip culture. *In Vitro Cellular & Developmental Biology-Plant*. 2005, **41**(2), 151-156. <https://doi.org/10.1079/IVP2004591>
- ÖZKAN, H. and AASIM, M. Potential of pretreated explants of peanut (*Arachis hypogaea* Linn.) to micropropagation under *in vitro* conditions. *Pakistan Journal of Agricultural Sciences*. 2019, **56**(3), 775-780. <https://doi.org/10.21162/PAKJAS/19.8262>
- PALANIVEL, S. and JAYABALAN, N. Direct multiple shoot induction from different mature seed explants of groundnut (*Arachis hypogaea* L.). *Philippine Journal of Science*. 2002, **131**(2), 127-135.
- PALANIVEL, S., et al. *Arachis hypogaea* L. direct organogenesis and SDS-PAGE analysis in groundnut. *British Biotechnology Journal*. 2014, **4**(6), 720-732. <https://doi.org/10.9734/BBJ/2014/9237>
- PEETERS, A.J.M., et al. *In vitro* flower bud formation in tobacco: Interaction of hormones. *Plant Physiology*. 1991, **97**(1), 402-408. <https://doi.org/10.1104/pp.97.1.402>
- PESTANA, M.C., et al. *In vitro* regeneration of peanut (*Arachis hypogaea* L.) through organogenesis: Effect of culture temperature and silver nitrate. *In Vitro Cellular & Developmental Biology-Plant*. 1999, **35**(3), 214-216. <https://doi.org/10.1007/s11627-999-0080-3>
- PRATAP, A., et al. Potential, constraints, and applications of *in vitro* methods in improving grain legumes. *Plant Breeding*. 2018, **137**(3), 235-249. <https://doi.org/10.1111/pbr.12590>
- RADHAKRISHNAN, T., et al. Micropropagation in peanut (*Arachis hypogaea* L.). *Biologia Plantarum*. 2000, **43**(3), 447-450. <https://doi.org/10.1023/A:1026743822546>
- RASOOL, R., et al. Effect of BAP and NAA on shoot regeneration in *Prunella vulgaris*. *Journal of Natural Sciences and Mathematics, Qassim University*. 2009, **3**(1), 21-26. ISSN 1658-4031.
- SAVAGE, G.P. and KEENAN, J.I., 1994. The composition and nutritive value of groundnut kernels. In: J. SMARTT, ed., *The groundnut crop: A scientific basis for improvement*, Dordrecht:Springer, pp. 173-213.
- SHARMA, K.K. and BHATNAGAR-MATHUR, P., 2006. Peanut (*Arachis hypogaea* L.). In: WANG, K., ed., *Methods in molecular biology, vol. 343: Agrobacterium Protocols, 2/e, volume 1*. New Jersey:Humana Press Inc., pp. 347-358.
- SUU, T.D., et al. *Peanut Planting and Care Techniques*. Ha Noi:Vietnam Academy of Agricultural Sciences - National Agricultural Extension Center, 2017.
- TIWARI, S. and TULI, R. Factors promoting efficient *in vitro* regeneration from de-embryonated cotyledon explants of *Arachis hypogaea* L. *Plant Cell, Tissue and Organ Culture*. 2008, **92**(1), 15-24. <https://doi.org/10.1007/s11240-007-9297-1>
- TORRES, K.C. *Tissue Culture Techniques for Horticultural Crops*. New York:Van Nostrand Reinhold, 1989.
- VASAVIRAMA, K. and KIRTI, P.B. Increased resistance to late leaf spot disease in transgenic peanut using a combination of PR genes. *Functional & Integrative Genomics*. 2012, **12**(4), 625-634. <https://doi.org/10.1007/s10142-012-0298-8>
- VENKATACHALAM, P., et al. Induction of direct somatic embryogenesis and plant regeneration from mature cotyledon explants of *Arachis hypogaea* L. *Current Science*. 1999, **77**(2), 269-273.
- VERMA, A., et al. Effects of *in vitro* triacontanol on growth, antioxidant enzymes, and photosynthetic characteristics in *Arachis hypogaea* L. *Brazilian Journal of Plant Physiology*. 2011, **23**(4), 271-277. <https://doi.org/10.1590/S1677-04202011000400004>
- VERMA, A., MALIK, C.P. and GUPTA, V.K. Sodium nitroprusside-mediated modulation of growth and antioxidant defense in the *in vitro* raised plantlets of peanut genotypes. *Peanut Science*. 2014, **41**(1), 25-31. <https://doi.org/10.3146/PS12-13.1>

Received: 20 August 2020 | Accepted: 21 October 2020 | Published: 27 April 2021



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