Rhizobox-Based Study of Chickpea (*Cicer arietinum* L.) Root System Architecture:

Standard Operating Procedure (SOP) for Phenotyping and Screening to Complement Pre-Breeding

Asnake Fikre (PhD)
Tulu Degefu (PhD)
Kasahun Admike (MSc)
Peter Doerner (PhD)

Technical Manual
No. 2/2019

ICRISAT
Science with a human face
International Crops Research Institute for the Semi-Arid Tropics

Rhizobox-Based Study of Chickpea (*Cicer arietinum* L.) Root System Architecture:

Standard Operating Procedure (SOP) for Phenotyping and Screening to Complement Pre-Breeding

Asnake Fikre (PhD)
Tulu Degefu (PhD)
Kasahun Admike (MSc)
Peter Doerner (PhD)

June, 2019
Website: https://www.icrisat.org//
Tel: +251-11-6-172540
Fax: +251-11 646 1252/646 4645
P.O.Box *ILRI (C/o) 5689*
Addis Ababa, Ethiopia

Contents

Rhizobox-Based Study of Chickpea (*Cicer arietinum* L.) Root System Architecture: Standard Operating Procedure (SOP) for Phenotyping and Screening to Complement Pre-Breeding

Asnake Fikre[1],Tulu Degefu[1],
Kasahun Admike[2] and Peter Doerner[2]

[1]Regional Chickpea Breeder, ICRISAT, Addis Ababa, Ethiopia. Po.Box ILRI
[1]Rhizobiologist, ICRISAT, Addis Ababa, Ethiopia. Po.Box ILRI (C/o) 5689
[2]Network Security Expert & Image processing consultant, EIAR, Debre Zeit, Ethiopia, Po.Box 32; [3]Institute for Molecular Plant Science; School of Biological Sciences, University of Edinburgh, Edinburgh, UK

Correspondence: A. Fikre@cgiar.org

1. Background

As a reflection of its importance in many plant functions including nutrient and water uptake, anchoring and mechanical support, as an interface between the plant and soil, root system architecture study has become a topic on its own in many research areas. Therefore, current research undertakings are geared towards unraveling the development and architecture of root systems with the premises that it holds important information towards its exploitation in any research program, which aims to increase plant performance and economic yield. Nevertheless, there is a well-established fact that Root System Architecture (RSA) varies among different plant species, and the variation is well implicated, even within the same species, which is governed by the plant genotype and environment. Furthermore, the inherent difficulty of studying the root system (because of soil opacity) makes them recognized as being 'the hidden half' (Waisel *et al*., 2005), and because of the aforementioned factors, and/or probably some other unforeseen reasons, RSA has traditionally been neglected by researchers (for example plant breeders) in terms of integrating it as an important selection criterion in crop improvement program.

Because of the irrefutable importance of roots, several efforts have been made to study RSA, for instance, X-ray-based technique (Morris *et al.*, 2017), which enable to capture the 3D picture of root system. However, the shortcoming associated with this technique is that it is slow and requires expensive equipment, which restricts its wide application in resource limited countries. Consequently, other techniques including gellan gum, transparent synthetic soil and hydroponics have been developed to visualize roots in the lab. However, the behaviour of root system growth in those systems are entirely different from the way roots are responding to abiotic and biotic factors when it is grown in the natural environment, thus affects RSA (Morris *et al*., 2018). Therefore, irrespective of different techniques (soil-based growth systems or rhizobox) used to study RSA, one would always expect a certain level of compromise between realistic growth conditions and root visibility.

The rhizobox-based platform to study RSA is useful in many different aspects, including testing of plant growth behaviour in waterlogging (Dresbøll *et al.*, 2013), low moisture stress (Durand *et al*., 2016) or contrasting nutrient availability conditions (Jin *et al.*, 2015). For example, several rhizobox-based approach allow observation of only a small fraction of the root system growing in 3D (Sanders and Brown 1978), while in 2D visualization a variable fraction of the entire root system is collapsed (Neumann *et al.,* 2009) making it difficult to capture all useful information associated with RSA parameters. Taken together, the substantial cost arising from use expensive components to construct rhizobox has led them to be selected against its utilization on a wider scale. In addition, many are geared towards transparent systems and require significant human intervention by highly-trained operators (Kuijken *et al.*, 2015).

In Ethiopia, efforts have been made to construct a customized and affordable soil-based rhizobox that can widely be used to study RSA in chickpea by combating the drawback (Thibaut *et al.*, 2019). A project 2018/19 among University of Edinburgh, ICRISAT-Ethiopia and EIAR has brought an initiative on RSA study, which would shade a new technical dimension in the national chickpea breeding endeavour. From our earlier research observation of the two generations, we have established an optimized operational procedure as called Standard

Operating Procedure (SOP), which can be followed by researchers working on the same areas.

2. Objective

This manual is developed with the objective of setting Standard Operating Procedure (SOP) as user guide manual for studying the root system architecture or screening chickpea lines for root traits in 2D using rhizobox structure, which is an affordable and robust phenotypic framework.

3. Rhizobox Construction

The rhizobox, frame structure to support plant root growth in 2D, is made by assembling the components described in Table 1 and Figure 1. The rhizoboxes is sized with the assumption that it holds a 6 mm thick layer of soil between a sheet of dark brown PVC (PVC; 1500 mm x 450 mm), and a glass pane of the same dimensions as the PVC backing for a total soil volume of ~3.7 dm^3. A 6 mm silicone strip spacer is used between the two planes. Two aluminium U-channels are used to assemble and hold the PVC and glass pane together from the two sides, and a metal rod (wire) is inserted into a folded piece of nylon mesh to bind two aluminium U-channels, close the bottom of the rhizobox, and it allows water movement in and out. Fig. 1 below shows top view of rhizobox (a), rhizobox components (b).

Figure 1. Showing top view of rhizobox diagram (a) and components of rhizobox (b)

4. Media Preparation and Rhizobox Filling

Naturally in Ethiopia, about 95% of chickpea is grown and produced on vertisol. A black soil (vertisol), with 65-70% clay proportion was sourced from Debre Zeit

Agricultural Research Center (DZARC) test station, grounded (after being dried well) and sieved using locally available sieving mesh (with 1.5mm x 1.5mm dimension). Similarly, a vermi-based compost (equivalent to forest soil) soil was sieved and added and homogenized into a sieved vertisol with a proportion of 20%. This is thoroughly mixed uniformly and wasoptimally made to improve the structure and texture to a stable, as the vertisol alone showed cracking (shrivelling) that has affected the growing root structure. The blended media with the vermicompost minimized the inherent shrivelling or cracking associated with vertisol nature.

4.1. Moistening

The optimized blended soil with vermicompost is loaded horizontally into the dark brown PVC of the rhizobox (Fig. 3). The soil is manually spread uniformly using hand support bar run over the surface.

For each rhizobox, 1.35 litre of tap water was gently sprayed over to 2.7 kg of blended soils. Uniformity of moistening the soil on 150mmx45mm space area is made using spray funnel which release fine droplets (Fig. 2). Still layering (thin layer soil then-spray then-cover the remaining soil then- spray) approach is a possibility to effectively spread the moisture and get even moistening across the board. This approach could be more effective but more cautionary. Finally, after adding the glass pane, the system is closed with the aluminium U-channel frame (Fig. 1 and see Table 1 for dimensions of the U-channel). See the procedural details below.

Figure 2. Moistening the soil using spray funnel

- 2.7 kg of the sieved and blended soil is loaded and uniformly spread over the dark brown PVC sheet with silicon strips (6mm thickness). Hint: the silicon strips were used to monitor the thickness of the soil while levelling it. (See Fig. 3 below Soil uniformly spread over the dark brown PVC sheet). Can be done by one or two people.

Figure 3. Sieved and blended soil uniformly loaded and spread over the dark brown PVC

- Then the blended soil is gently moist up with 1.35 litter of water using locally available and customized funnel. NB: Misting is a must to avoid disturbance of uniformly spread soil on the pane in the rhizobox,
- Then after filling the soil and moistening it up, the glass pane is laid over the PVC, and held together

from the two sides using a U-channel aluminium, two people would do it better,

- Then, a metal rod (locker) is inserted into a folded piece of nylon mesh to fix the assemblage and close the bottom of the rhizobox,
- The upper parts of each rhizobox is held together using rubber plastic to avoid sudden shock damage.

5. Rhizobox Support Stand

- The rhizobox stand (Fig. 4) is a structure to support the rhizoboxes. The optimal angle of rhizobox laid against the structure is 450, which was found to maximize root appearance against the transparent glasses on the lower side. The stand has a dimension of 150cm x 45cm x 1.9cm in length, width and height. It is iron cast that can support many rhizoboxes with one laid over the other as shown in Figure 5 below.The rhizoboxes are kept on rhizobox supporting structure (Table 2) with inclination of 45 degree as shown on Fig. 4, for planting under which there was a plastic sheet filled with water having 6-7cm depth. NB:

rhizoboxes are put on one another using a flat cork (4-5 cm thick foam) in between the rhizoboxes to protect any possible damages arising from shocking.

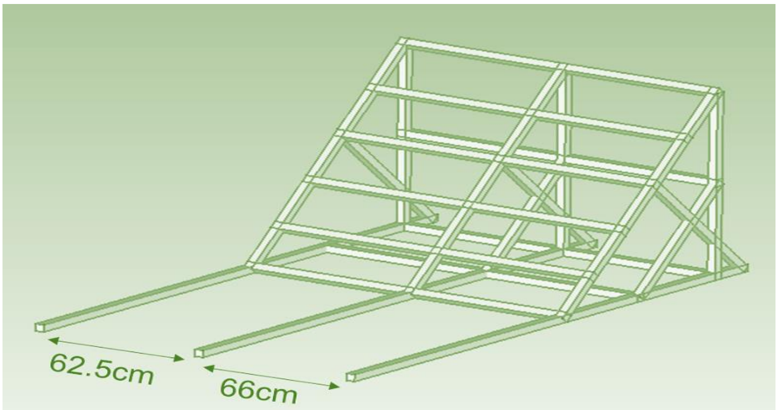

Figure 4. Rhizobox stand structure

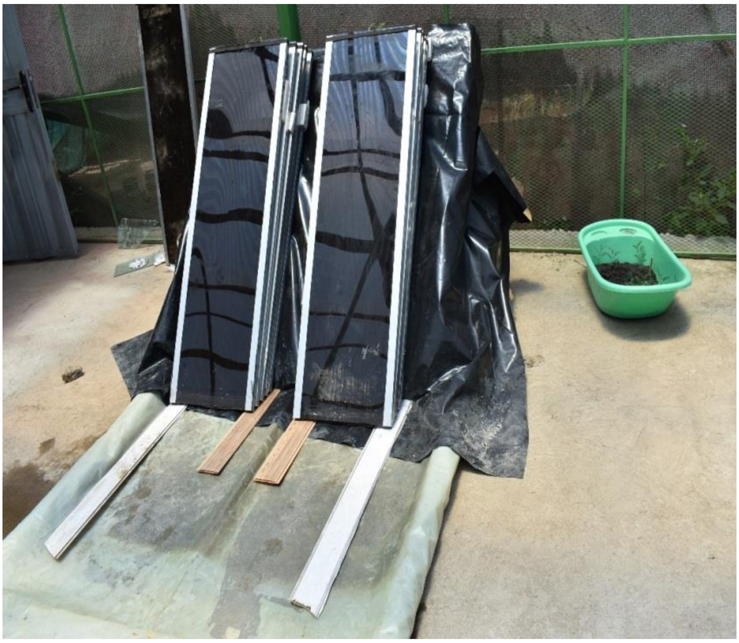

Figure 5. Many rhizoboxes laid over the other on rhizobox stand

6. Seed Planting and Plant Establishment

Seedling transplanting (b/n 6-8 days, with 4-6cm root and shoot length) is found effective to establish than direct seeding technique. Hence, seedling plot/pot would be necessity to temporarily serve as source of plantation. Practical experience in this regard have been mainstreamed as part of the procedure to better equip the methodology (See figure 6a and 6b). Technical details of procedure are presented below.

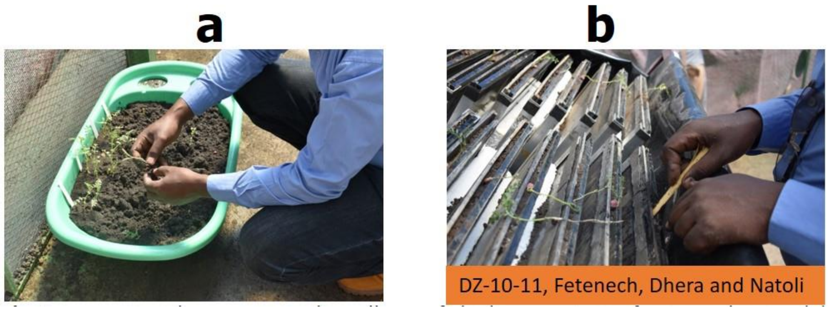

Figure 6. Uprooting the germinated seedlings of chickpea varieties for transplanting (a) and planting in the rhizobox (b)

- *Chickpea varieties were selected for the experiment*
- *Then the selected varieties were treated with Apron star to get rid of any possible fungal pathogens*
- *Right after treating the varieties, they were planted on a plastic jar filled with vertisol (moistened with water) sourced from DZARC for ex-situ germination*
- *Then upon the emergence of the first radicle, the seedlings (about a week old) were transplanted from the plastic jar to the rhizobox (Fig. 6b). NB: we initially transplanted two seedlings per rhizobox, and finally thinned down to one after making sure that the seedling was successfully established. NB: rhizobox preparation and the seedling emergence date should be well synchronized, thus transplanting was done immediately after rhizobox preparation to protect possible moisture loss through evaporation*
- *While planting, a hole (to a depth of 3-4cm) was made in the blended soil within the rhizobox with thin stick to accommodate root structure of the transplanted seedling,*
- *Then the entire rhizoboxes were covered with a black plastic sheet to mimic the underground natural soil where roots are supposed to grow in complete darkness. NB: In addition to mimicking the underground soil conditions, the black plastic sheet helps to selectively inhibit the growth of cyanobacteria, which in a normal circumstance require sunlight for their photosynthesis,*
- *For the sake of data management and handling, each rhizoboxes were labelled with marker as needed,*
- *Each rhizobox was laid on one another using a separator (shock absorber cork), and the rhizoboxes were arranged in such a way that the PVC side facing up, while the glass pen facing down, this favours roots growth habit against the glass due to gravitational force. (See fig. 7)*

Figure 7. Rhizobox laid on one another using a separator (Stirofoam)

- *The whole system was wrapped with reflective aluminium sheet (Polystyrene sheets, Table 2) to insulate against excessive heating from direct sunlight.*
- *The bottom side of the rhizobox was socked in 4-5 cm deep water bath*
- *Water was added to the plastic sheet at the base of the system to keep percolation and regularly monitored to maintain a constant water supply; another option is to grow in no water bath system to see drought responses.*

Output: robust and affordable system to study root system architecture has been developed from locally available materials at DZARC. This tool can be adapted and be used in research institutions, which aim to study root systems of chickpea, and possibly other crops. From our research undertaking, it was possible to conclude that different chickpea genotypes responded differently in terms of root growth, and that will have an implication on nutrient and water extraction from the soils.

7. Camera Support and Shooting System

Camera support system is a designed to facilitate progressive picturing and uploading to the system. It is anchoring multiple camera berry operating in an interconnected scheme of developing imaging. Intentionally it is a camera monitoring system through computational arithmetic's of the algorithm. It is a semi-automated set up where imaging is connected to a server system for analysis. The connected computer for logging would serve to visualize imaging and make any adjustment as needed for precision.

To this effect, each rhizobox, which is laid down on the rhizobox support shall be moved to the camera support for image shooting in every other days and back to their place. Two people are mobilizing the rhizoboxes for imaging with all possible cares not to make any damage out of the shock. Fig. 8 below showing camera support with dimensions labelled in number for which the length of the dimensions are indicated.

Piece Number	Length (mm)
1, 2	1500
3, 4, 5, 6	1660
7, 8	725
9, 10, 11, 12, 13, 14, 15, 16	680
17, 18, 19, 20, 21, 22	200
23, 24	945
25, 26	810
27, 28	100
29, 30	1510

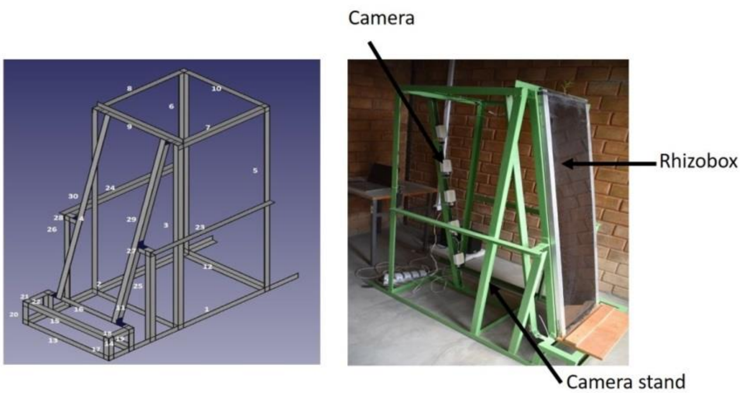

Figure 8. Camera support and length of each piece in (mm)

8. Image Station

Imaging station is a place where images of the roots through the transplant glass side will be progressively taken. Therefore, we tried to construct image station and rhizobox shelter together to keep proximity. The imaging station is constructed under secured, cool, dry and clean environment to protect the camera from unintended malfunctioning damage. (See Figure. 9).

Figure 9. Image station in rhizobox shelter

9. Root Parameters to be Measured/Extrapolated and Possible Application

The soil-based growth container (rhizobox) along with the imaging system is assumed to phenotype root development. Root system architecture (RSA), defined as the spatial distribution of plant roots in the soil, is an emergent property that includes additional parameters to describe the spatial interaction of roots with their environment. Quantitative phenotyping using auto-/semi-automated imaging will make it possible to explore the link among different processes like nodulation patterning, root system architecture, and plant yield. Root architecture and root morphology (e.g. root diameter) or topology (e.g. number and hierarchy of secondary roots). In the present system key anlytical detrminant parameters to be measured using the includes, Root density, Root length, Root biomass, Root volume/convex hull area/ using camera imaging technique. RSA parameters reflect soil resource acquisition strategies: root system length and area describe the individual investment in root biomass for resource foraging and acquisition. Root solidity (ratio root/convex hull areas) reflects a trade-off between foraging and space occupancy. It provides insight into the strategy to acquire soil resources: extend or intensify, with higher solidity reflecting more intense resource foraging within the explored area. Deep rooted growth and spatial/lateral based growth of the root structure have two different resource foraging nature, where the first is more on moisture the second more on nutrient resources.

Hence, using the technique germplasm lines could be characterized as a re-breeding inputs of the improvement program. This would be challenging to handle large number, however, complementarity based key germline testing and describing potential clustering/eg identification of drought response traits etc/ be a future possibility platform.

Figure 10. Pictures demonstrating root system architecture phenotyping, potential as pre-breeding support system

10. Appendix: Component Specification and Description

Appendix 1. Rhizobox components specification and description

No.	Parts	Specification/Dimensions	Source/supplier
1	Glass sheet	150cmx45cmx0.8cm	Local Market, Addis Ababa, Ethiopia
2	Dark brown PVC sheet	150cmx45cm x0.6cm	Local Market, Addis Ababa, Ethiopia
3	Silicone Spacer strips	150cmx1cmx0.6cm	Silex Silicones (Lindford, UK)
4	Nylon mesh	1.8m	Local Market, Bishoftu, Ethiopia
5	Metal rod	Stainless steel 67cm	Local Market, Bishoftu, Ethiopia
6	Aluminium U-channels	• 150cmx2.4cmx2.5cm • Thickness 0.2cm • Net internal size 2.0cm x 2.2cm	Local Market, Addis Ababa, Ethiopia
7	Soil	Vertisol	DZARC
8	Compost	Vermicompost	DZARC

Appendix 2. Support components for rhizobox

#	Component	Dimensions (cm):	Supplier
1	Angle iron		Local Market, Addis Ababa, Ethiopia
2	Metal foot		Local Market, Addis Ababa, Ethiopia
3	Thick plastic sheet for holding water		Local Market, Addis Ababa, Ethiopia
4	Spacer b/n rhizobox		DZARC, Ethiopia
5	Polystyrene sheets, ECO100 grade		Custompac (Castleford, UK)
6	Black plastic sheet		Local Market, Bishoftu, Ethiopia

Appendix 3. Imaging station

Component	Dimensions (cm)	Supplier
Angle iron		Bishoftu, Ethiopia
Metal feet		
LED strip (natural white colour, 4.8 $W.m^{-1}$)	150	
LED power supply with dimmer (12 V, 2 A)		Powerpax (UK)
Raspberry PI 3 Model B V1.2		Farnell (UK)
16GB MicroSD Card with preloaded OS Installer		
Official Raspberry Pi International PSU		
Cameras (Raspberry Pi 3 model B V1.2)		Waveshare (US)

11. References

Dresbøll, D., Thorup-Kristensen, K., Mckenzie, B., Dupuy, L. and Bengough, A. (2013) Timelapse scanning reveals spatial variation in tomato (Solanum lycopersicum L.) root elongation rates during partial waterlogging. *Plant Soil*, 369, 467–477.

Durand, M., Porcheron, B., Hennion, N., Maurousset, L., Lemoine, R. and Pourtau, N. (2016)

Jin, K., Shen, J., Ashton, R., White, R., Dodd, I., Parry, M. and Whalley, W. (2015) Wheat root growth responses to horizontal stratification of fertiliser in a water-limited environment. *PlantSoil*, 386, 77-88.

Kuijken, R.C., van Eeuwijk, F.A., Marcelis, L.F. and Bouwmeester, H.J. (2015) Root phenotyping: from component trait in the lab to breeding. *J Exp Bot*, 66, 5389-5401.

Morris, E.C., Griffiths, M., Golebiowska, A., Mairhofer, S., Burr-Hersey, J., Goh, T., von Wangenheim, D., Atkinson, B., Sturrock, C.J., Lynch, J.P., Vissenberg, K., Ritz, K., Wells, D.M., Mooney, S.J. and Bennett, M.J. (2017) Shaping 3D Root System Architecture. *Curr Biol*, 27, R919-R930.

Morris, J.L., Puttick, M.N., Clark, J.W., Edwards, D., Kenrick, P., Pressel, S., Wellman, C.H., Yang, Z.H., Schneider, H. and Donoghue, P.C.J. (2018) The timescale of early land plant evolution. *P Natl Acad Sci USA*, 115, E2274-E2283.

Neumann, G., George, T.S. and Plassard, C. (2009) Strategies and methods for studying the rhizosphere-the plant science toolbox. *Plant and Soil*, 321, 431-456.

Sanders, J.L. and Brown, D.A. (1978) New Fiber Optic Technique for Measuring Root-Growth of Soybeans under Field Conditions. *Agron J*, 70, 1073-1076.

Thibaut Bontpart, Cristobal Concha, Valerio Giuffrida, Ingrid Robertson, Kassahun Admkie, Tulu Degefu, Nigusie Girma, Kassahun Tesfaye, Teklehaimanot Haileselassie, Asnake Fikre, Masresha Fetene, Sotirios A. Tsaftaris and Peter Doerner (2019). Affordable and robust phenotyping framework to analyse root system architecture of soil-grown plants. https://www.biorxiv.org/content/10.1101/573139v1

Waisel Y., Eshel A., Beeckman T., Kafkaki U. (2005). Plant roots: the hidden half, 1st edn. New York, NY: Marcel Dekker [Google Scholar]