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Method Article

“ASURE”: A multi-potential plant bioassay as a pre-determinative microbial efficiency testing tool for bioinoculant studies



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A B S T R A C T

Bioinoculant studies focusing on plant-microbe association hold immense importance for research in field of agriculture and plant science. Such plant-microbe associations are amongst the most complex and beneficial partnerships in nature. Research focused to explore favourable host-microbe relationship requires a contamination free, non-interceptive and easily manageable system where these interactions can be studied in real-time. Also a provision for efficient sample recovery to support a variety of analyses would be a definite advantage. The manuscript proposes a new **multi-potential plant bioassay abbreviated “ASURE”** inspired by hydroponics. ASURE is a robust system, providing a workable solution to challenges faced during in-vitro microcosm studies. Significance of this system is its plant growth supporting design, facilitating comparative assessment of PGPM treatment benefits to host plant while providing a closer real-time view of plant microbe association.

- ASURE serves as a testing tool to pre-determine the efficiency of various plant friendly microbes to develop them as future bioinoculants.
- ASURE accommodates 16 individual samples in a 12 × 12 cm space, ensures homogenous micro environment and facilitates continuous and undisturbed (real-time) monitoring of root and shoot growth.
- ASURE enables efficient sample recovery with zero loss during harvesting and no interception from substrate.

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A R T I C L E I N F O

Keywords: Plant growth promoting bacteria, PGPM, Trichoderma, Rhizobacteria, Plant bioassay, Bioinoculants

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Specification Table

Subject Area:	Agricultural and Biological Sciences
More specific subject area:	Environmental Microbiology
Method name:	ASURE
Name and reference of original method:	Tube-in-tube method Mishra, N., Sundari, S.K., 2017. A "six-step-strategy" to evaluate competence of plant growth promoting microbial consortia. <i>Curr. Sci.</i> 113, 63–70. https://doi.org/10.18520/cs/v113/i01/63-70
Resource availability:	All information included in the article. No additional resource requirement (software/hardware) No additional data.

Method details

Background

In order to identify most beneficial plant microbe combinations, researchers conduct host inoculation studies by treating selected host plants with a variety of PGPM and assess their efficiency. These efficacy studies were generally carried out in 3 different ways (a) in-vitro seed germination assays (paper towel method, water agar, moist cotton based) (b) in-vitro agar slants, plastic pouches or tray assays of max 1 week duration and (c) pot trials extending between 1–3 months in polyhouse/greenhouse [1–10]. Based on results from above suggested methods, few studies further proceed to field trials and propose successful PGPM isolates as probable bio-fertilizer/biopesticide/bioinoculant. Existing systems for in-vitro studies face challenges in maintaining homogeneity, microbial inoculum stability, managing large number of replicates and optimizing microbe's colonization efficiency. Moreover, method dependent variations crop at multiple stages viz., in maintaining sterile/semi-sterile conditions, choice of soil/soil-like/agar/liquid substrate, feasibility to manage ample number of replicates for every treatment, sample loss during harvest (particularly using soil/soil like substrate), wide variation in period of incubation (ranging from 3 days to 3 months @ method used) and so on. Such dissimilarities bring about considerable disparity in data which makes comparative assessment and interpretation between different studies impractical and may at-times lead to false/biased interpretations. Table 1 is a concise representation where all the positive attributes of ASURE are presented in comparison to other plant bioassays periodically employed to evaluate inoculation benefits of PGP microbes.

Design improvisation for bioinoculant studies

Present method is a leap ahead of earlier published research from author's laboratory [16] where PGP indicative parameters were studied in a tube-in-tube system exclusively for bacterial (PGPR) inoculation. While tube-in-tube was appropriate for short term studies, it presented certain difficulties. For fast growing hosts like Sorghum or Vigna, the maximum period of incubation can be up to 10 days in tube-in-tube method, beyond which growing roots would face space limitation for proper growth. Moreover, tube-in-tube can hold one plantlet per setup. As every treatment had to be replicated to a minimum of 9 replicates, as many number of setups have to be added, increasing chances for inter-replicate variability of in-vitro micro-environment in individual tubes. In tube-in-tube setup, the lower edge of the tailored eppendorf touches the bottom of the outer test tube presenting two difficulties: a) addition of more substrate causes submergence of the seed, which in turn limits the substrate volume and caps the incubation period to 10 days max., depending on plant host used and b) the emerging roots in tube-in-tube setup have to nudge the lower end of the eppendorf to emerge and establish which may cause injury in young and delicate secondary roots. ASURE circumvents all these issues. ASURE holds 16 replicates for single treatment ensuring inter-replicate homogeneity while providing ample room for the growing roots for extended incubation period (Table 1).

Table 1

Positive attributes of ASURE over other plant bioassays.

	Other Methods				Base Method	Proposed Method
	<i>In vitro</i> Petri plates (Horizontal/vertical) based method	<i>In vitro</i> Tubes/bottles/ flasks based assays	<i>In vivo</i> Pot experiments	<i>In vitro</i> Hydroponics	<i>In-vitro</i> Tube-in-tube	ASURE
Space for experimental setup-shelf/floor area	a).9-12 cm/plate-horizonal 12.5 × 12.5 × 1.5 cm/ plate-Vertical b).Accommodates mostly 1 or at the max 2 replicates / plate/ treatment.	a).1.3 cm x 10 cm; tube 1.6 cm x 15 cm; tube 2.0 cm x 15 cm tube b).Accommodates 1 replicate/tube/ treatment.	a).6-12 inches/pot; 15.24 cm × 30.48 cm/ pot b).Accommodates 1 replicate/pot/ treatment.	a).10.7 cm × 10.7 × 5 cm per unit/treatment b).Accommodates 1-15 replicates/ unit/ treatment.	a).2.0 cm x 15 cm per unit per treatment b). Accommodates 1 replicate /tube/ treatment.	a).12 cm × 12 cm per unit/treatment b).Accommodates 16 replicates/ unit/ treatment. c).Provides maximum space economy.
Media and quality control	a).Agar based media. Aseptic conditions maintained. b).Not possible to replenish growth media. c).Not suitable for long term studies. As incubation proceeds, use of agar media may give rise to unequal accumulation of media components and growth metabolites.	a).Either liquid or semi solid agar media employed. b). Though media replenishment possible where liquid media is used. c).It may prove to be laborious to deal tube/ flask by flask individually and excessive handling may invite contamination.	a).Pre sterilized soil/ soil like substrate used. No aseptic condition during incubation. Risk of nutrient loss due to leaching. b).Possibility to provide additional nutrients / media during watering. c).Might invite contamination due to extraneous microbes, which in turn prey upon media & nutrients.	a).Aseptic conditions maintained throughout b).Replenishment of media is possible c).Higher chances of contamination through water/air pipes	a).Liquid media used. Aseptic conditions maintained throughout. b).No media replenishment done. c). Zero possibility of contamination	a).Aseptic conditions maintained throughout. b).Media replenishment can be done as needed with minimal intrusion. c). No contamination issues d).Liquid condition increases accessibility of nutrients to all replicates during incubation.
Level of complexity	a).Setup is simple but becomes laborious to accommodate individual units for every replicate, particularly when large numbers of treatments are involved. b).Results obtained will be very basic, as actual impact of plant microbe	a).Comparatively laborious and difficult to handle large number of treatments	a).Very laborious and require regular watering and leachate management. b).Study can be extended even upto 4 months giving advanced plant growth data c). Risk of incidence of pests during incubation	a).Very laborious and require regular watering	a).Comparatively laborious and difficult to handle large number of treatments	a).Extremely easy to manoeuvre and practically hassle free set up.

Table 1 (Continued)

	Other Methods				Base Method	Proposed Method
	<i>In vitro</i> Petri plates (Horizontal/vertical) based method	<i>In vitro</i> Tubes/bottles/ flasks based assays	<i>In vivo</i> Pot experiments	<i>In vitro</i> Hydroponics	<i>In-vitro</i> Tube-in-tube	ASURE
Uniformity	association mostly emerges over a period of maturation. a).Non-uniform in terms of substrate and its microbial inoculum distribution b).Leaves room for chances of variation amongst sample replicates within a specific treatment. c). Non-uniformity of micro-environment cannot be ruled out.	a).Non-uniform in terms of substrate and microbial inoculum distribution (upon use of solid media) b).Leaves room for chances of variation amongst sample replicates within a specific treatment. c).Non-uniformity of micro-environment cannot be ruled out.	a).Heterogeneous. Risk of formation of nutrient pockets inaccessible for growing roots/ microbes. b).Intra-replicate variability within treatments is high c). Non-uniformity of micro-environment. Hence large number of replicates to be studied for viable data interpretation.	a).Uniform both in terms of media and micro-environment	a).Uniform in terms of nutrient availability through media and micro-environment. b).Risk of inter-replicate variability	a).Uniform and homogenous setup. b).No inter-replicate variability. c).No variation of micro- environment among sample replicates.
Duration of experimentation	Diverse incubation periods: 3/5/7 days.	Diverse incubation periods ranging from as little as 3 days up to 15 days in rare conditions.	3 weeks (<i>In-vitro</i>) 3 months to complete season (<i>In-vivo</i>).	15 days for bioinoculant studies.	Max.10 days for bioinoculant studies.	Minimum 2 weeks extendable as per experiment requirement.
Speed to Harvest	a).Rapid recovery but data obtained has limited significance. b). Sample loss takes place during harvest.	a). Moderate time for harvest. b).Chances of sample loss (for solid media) during harvest.	a).Delayed harvest due to extended period of incubation. b).High chances of sample loss during harvest.	a).Moderate time for harvest b).Zero sample loss at the time of harvest.	a).Moderate time for harvest. b).Harvesting is laborious due to the setup	a).Rapid recovery b).Zero sample loss at the time of harvest.
Growth parameters studied	Dry weight Root growth Seed vigour testing, Germination percentage calculation.	Dry weight Root growth Seed vigour testing, Germination percentage calculation Secondary metabolites and plant growth regulators study.	RL, SL, DW, Chlorophyll content and protein, Root shoot ratio @ Seasonal studies: branching, flowering and yield.	RL, SL, DW, Chlorophyll content and protein.	Dry weight Root growth testing, Secondary metabolites and plant growth regulators study.	RL,SL, DW, Chlorophyll content, protein, SEM analysis, Secondary metabolites and plant growth regulators study

Applicability as pre-determinative tool for bioinoculant studies

a).Economical but short term and hence require further experimental proof to determine PGP isolates capabilities.

a).Economical but require frequent plant transfer to fresh media for extended bioinoculant studies.

a).Costly, laborious, time consuming, requires special infrastructure like green-house/ poly-house.
b).Not suitable for root architecture studies.

a).Very Costlier compared to other system as each setup can hold mostly single or limited treatments. b). Requires specific infrastructure /equipment, electrical supply.

a).Economical but require frequent plant transfer to fresh media for extended bioinoculant studies.

a).Aseptic, Simple, Uniform, Rapid Economical (ASURE).
b).Suitable for root architecture studies. c). Applicability for bacteria, consortia and fungal bioinoculant studies including media optimization studies, stress resistance studies, pollutant degradation studies, Secondary metabolites and hormone quantification studies.
Current Article

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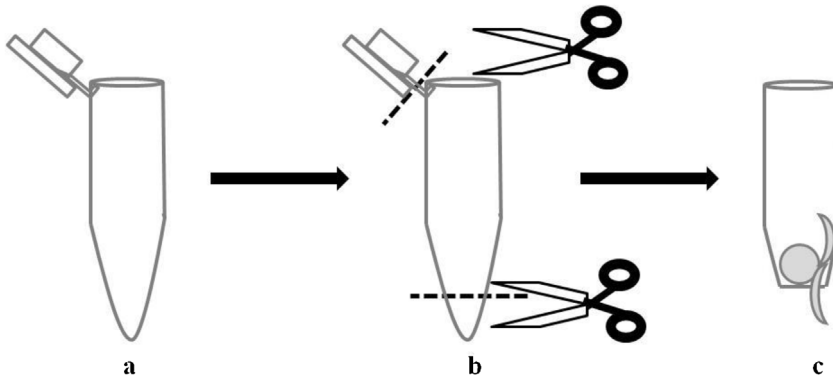
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- a) Image of 1.5 ml eppendorf b) Trimming eppendorf as shown c) Customised eppendorf used in experiment

Fig. 1. Customizing eppendorf for ASURE experimental setup.

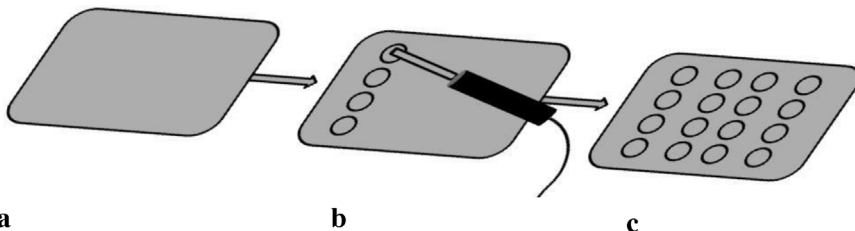
a) Top view of box lid b) Drill specific size apertures using soldering iron c) final look of the box lid with equally spaced apertures to hold eppendorf tubes.

Media used in the study were: Hoagland media (Himedia, India), minimal media (MNM) as defined by Roza et al. [17] and Plant microbe bioassay (PMB) medium developed in laboratory. PMB contained $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, NaCl , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, KH_2PO_4 , FeCl_3 , $(\text{NH}_4)_2\text{HPO}_4$ and sodium citrate. Concentration of salts $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was modified from MNM to 0.05 g/l and 0.001 g/l respectively, to avoid precipitation of salts. Technical grade monocrotophos (36% pure) used in the study was procured from Jai Shree Rasayan Udyog Ltd., Nathupur, Haryana. Carboxyl methyl cellulose (Himedia) was used for seed bio priming during fungal treatment. Other materials used for the experiment included commercial microwaveable plastic boxes and autoclaved eppendorf tubes.

Reagents and media

ASURE setup preparation

- 1 To design a customised ASURE setup for the experiment, take a microwaveable rectangular plastic box.
 - 2 Trim the eppendorf tube (1.5 ml) at two places as shown in Fig. 1.
 - 3 Drill equal sized apertures on the lid of the plastic box using soldering iron to hold a total of 16 eppendorfs. Schematic representing process for customizing the box lid is shown in Fig. 2.
- Note: Take Ample care to maintain equal diameter of the apertures and uniform spacing between apertures.



- a) Top view of box lid b) Drill specific size apertures using soldering iron c) final look of the box lid with equally spaced apertures to hold eppendorf tubes

Fig. 2. Customising top lid of the box for ASURE setup.

4 Position customised eppendorfs in the apertures avoiding any air spaces. Wrap the entire setup in autoclavable bag and then autoclave.

ASURE method

- 1 Carefully handpick healthy seeds of host plant (Sorghum variety RVJ-1862 used in present study) for uniformity, remove any damaged/infected seeds before soaking in luke warm water, overnight.
- 2 Perform surface sterilization of pre-soaked seeds as given in Mishra & Sundari [16].
- 3 Place sterilized seeds on 0.25% water agar using sterile forceps and incubate for 2 days.
Note: Cover the petriplate with brown paper to maintain dark condition for seed germination and monitor seed growth periodically.
- 4 Prepare microbial inoculum (MI)/bioinoculant (bacteria/fungi) as per the desired objective of the experiment.
Note 1: Microbial inoculum, containing $\sim 10^8$ cells/ml saline was used.
Note 2: Saline recommended for MI preparation to avoid reminiscence of other media salts.
- 5 Treat the germinated seeds of host plant with respective MI in pre-sterilised conical flask and incubate at 30 °C with occasional shaking for ~ 1 h.
- 6 After seed dressing, place the MI coated seeds in pre-sterilized, tailored eppendorf as shown in Fig. 1. Similarly sterile saline treated (MI untreated) seeds can be placed in a different box for control.
- 7 Add 300 ml of respective media to the box ensuring that its level touches the lower tip of tailored eppendorf.
Note: MI suspension can also be added at the time of initiating incubation.
- 8 Wrap the lower portion of the box with aluminium foil (to provide dark condition to growing roots) and then incubate the entire setup for 15 days.
Note 1: Incubation period can be varied as per requirement.
Note 2: For extended incubation (>15 days) replenishment of media can be easily done under aseptic conditions without hampering plant growth.
- 9 Post incubation, harvest plants and analyse for PGP indicative parameters such as root length (RL), shoot length (SL), dry weight (DW), chlorophyll (Chl) content, carotenoids (Car) and total protein content. Results from the analyses can be tabulated (Table 2 and Fig. 3).

Media optimization for "ASURE"

Media optimization studies were conducted to check the most appropriate medium to support plant growth and bacterial growth simultaneously in the given conditions. Rhizobacterial isolate RB3 used as bacterial inoculum, hereon referred to as BI. Five different media treatments namely: Hoagland with BI (H + BI) and without BI (H); $1/2$ strength Hoagland with BI ($1/2$ H + BI) and without BI ($1/2$ H); $1/2$ strength Minimal media with BI ($1/2$ MNM + BI) and without BI ($1/2$ MNM); Full strength PMB

Table 2
Impact of microbial treatment on studied plant growth parameters.

Parameters Studied	Control	Host + BI
Root length (cm)	3.97 ^a ±0.15	9.93 ^b ±0.4
Shoot length (cm)	6.13 ^a ±0.11	8.0 ^b ±0.1
Dry weight (mg)	20 ^a ±0.002	30 ^b ±0.0002
Chl a (mg/gm FW)	0.591 ^a ±0.003	0.817 ^b ±0.0013
Chl b (mg/gm FW)	0.234 ^a ±0.0054	0.272 ^{ab} ±0.0016
Total Chl (mg/gm FW)	0.829 ^a ±0.008	1.090 ^b ±0.002
Carotenoids (mg/gm FW)	0.266 ^a ±0.0014	0.304 ^b ±0.014
Total Protein (mg/gm FW)	0.956 ^a ±0.006	2.14 ^b ±0.005

FW = fresh weight; Standard deviation (SD) is mentioned with a sign \pm in the table for all the samples; ^{a, b} shows significant difference (p value < 0.01); ^{ab} shows difference but non-significant.

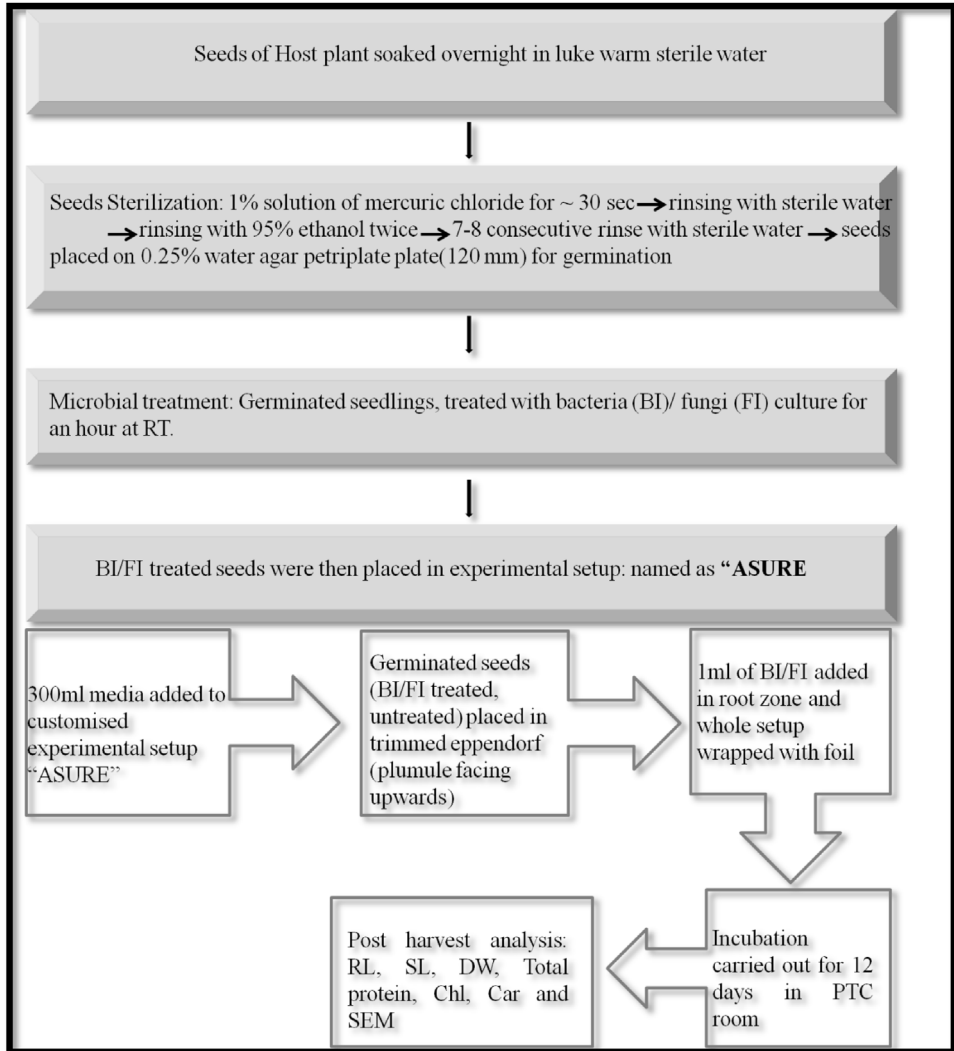


Fig. 3. Quick overview of steps to be followed for placing **Plant bioassay: “ASURE”**.

with BI (PMB 2X + BI) and without BI (PMB 2X); $\frac{1}{2}$ strength PMB with BI (PMB + BI) and without BI (PMB) were placed. Seed dressing and experimental box setup were done as explained in ASURE setup. Incubation was carried out for 10 days. Post harvest analysis included evaluation of plant growth (total plant biomass) and bacterial growth (cfu/ml).

Application of “ASURE” to study tolerance towards organic pollutant

The organic pollutant used in the present study was pesticide monocrotophos at concentration 300 ppm. The study included two treatments: stressed plant (host + pesticide MCP), stressed plant treated with BI (Plant + MCP + BI) and a healthy plant as control. Seed treatment with BI was performed as mentioned in methodology section. 300 ml of PMB media was used for all the

Table 3

Growth parameters recorded on applying ASURE for bioremediation studies.

Parameters Studied	Healthy Control	Stressed plant	Stressed plant + BI
Root length (cm)	3.97 ^a ±0.15	3.133 ^b ±0.15	3.967 ^a ±0.052
Shoot length (cm)	6.13 ^a ±0.11	2.067 ^b ±0.12	1.960 ^b ±0.057
Dry weight (µg)	20 ^a ±0.002	13 ^b ±0.001	30 ^c ±0.001
Chl a (mg/gm FW)	0.591 ^a ±0.003	0.234 ^b ±0.006	0.866 ^c ±0.004
Chl b (mg/gm FW)	0.234 ^a ±0.0054	0.057 ^b ±0.002	0.272 ^a ±0.007
Total Chl (mg/gm FW)	0.829 ^a ±0.008	0.289 ^b ±0.006	1.137 ^a ±0.010
Carotenoids (mg/gm FW)	0.266 ^a ±0.0014	0.104 ^b ±0.002	0.341 ^c ±0.007

Control = Untreated/ pesticide stressed control, Standard deviation (SD) with a sign ± is mentioned in the table; ^a, ^b shows significant difference (p value < 0.01).

treatments. Post incubation (15 days), samples were harvested and plant growth parameters namely: SL, RL, DW, Chlorophyll (Chl) and Carotenoids were studied and results presented in Table 3.

Statistical analysis

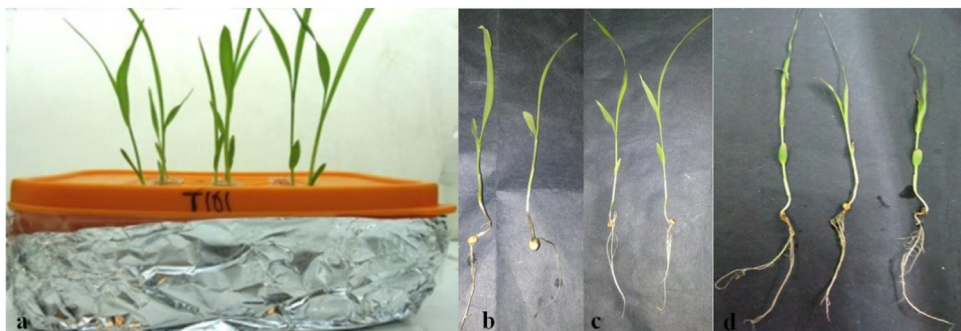
Student *t*-test was performed to calculate significant difference (at p value <0.01) between untreated control and microbe treated samples. Microsoft office Excel (MS) 2013 software was used for the purposes.

Method validation

“ASURE” as a pre-determinative tool

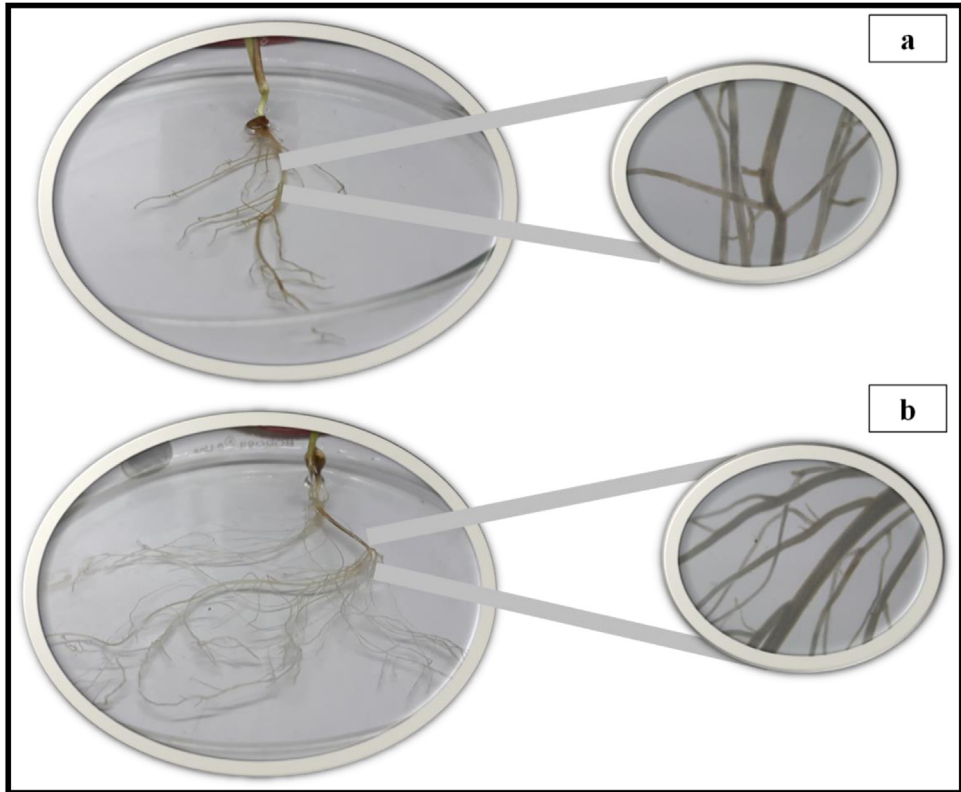
“ASURE” bioassay was performed to evaluate plant growth promotion ability of rhizobacterial isolate RB3 (BI) with host plant Sorghum (variety RVJ-1862). Parameters indicative of growth were compared and contrasted between BI inoculated and un-inoculated host plant in ASURE setup. As evident from Table 2, BI inoculated host has recorded increment in each and every growth parameter studied.

Based on measured growth parameters, host plant treated with BI showed enhanced shoot length (30.4%), root length (115.9%) and over all biomass (48.17%) as compared to untreated control (Fig. 4b, c). There was also a substantial increase in branching for BI treated roots (Fig. 5). BI inoculation resulted in 32% increase in total Chlorophyll (39% ↑ in Chl a and 23% ↑ in Chl b) and 14% ↑ in Carotenoids as compared to control. Protein content showed a phenomenal increase (110%) as compared to un-inoculated control. Total plant protein is a reflection of overall plant growth and thus such an increase in total protein content confirms the positive benefits accrued due to inoculation with BI. Earlier



a) ASURE setup; b) Untreated control; c) BI inoculated host; d) FI treated host plant

Fig. 4. Impact of bioinoculant on host plant growth applying ASURE setup.



a) Control root architecture; b) BI treated root architecture

Fig. 5. Sorghum root branching and architecture in BI untreated and BI treated host.

studies that targeted plant growth with PGP inoculation have also concentrated on these five parameters as reliable parameters for comparing the efficiency of one treatment over the other [3–5,11,18].

Post harvest SEM image of BI inoculated host plant root presents a clear evidence of a healthy association between sorghum and rhizobacterial isolate RB3 (Fig. 6a). Such an association between PGPM and host plant is known to uphold several benefits such as biocontrol, rhizoremediation and exchange of growth supporting metabolites including growth hormones [19].

ASURE can also be easily replicated to check upon inoculation benefits using different isolates simultaneously. ASURE would function as a most reliable tool for screening and selection of any number of isolates in most economical and scientifically verifiable manner yielding statistically viable growth data in as little as two weeks' time. ASURE precisely fits the role of 'a pre-determinative' tool before proceeding for large scale greenhouse trails with potential PGPR candidates that can be developed as bioinoculants in future.

Applications of ASURE: Impact of different media on host and microbe

ASURE can also be applied for media optimization studies and studies targeted towards checking the effect of abiotic/biotic stress on plants and impact of PGPR as bioinoculant on host plant under stress. Authors attempted such a study and presented the outcome in Fig. 7.

Fig. 7 reveals that Hoagland's did not support microbial growth to the same extent as PMB media. Similarly between PMB and PMB 2X, plant growth was surely better with PMB though bacterial

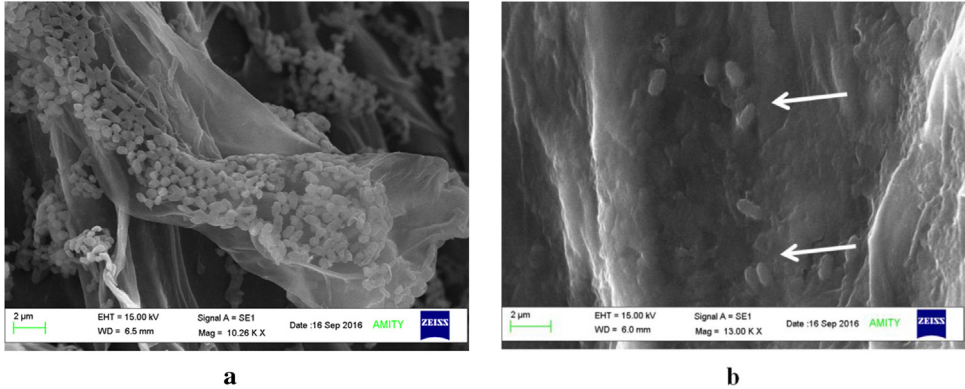


Fig. 6. SEM images of sorghum and BI association using (a) ASURE versus (b) Tube-in-Tube method [16].

growth did not show marked difference. PMB fared well both in terms of supporting plant growth (DW values) and microbial growth (cfu/ml), thus becoming the chosen media in all our plant bioassay studies.

Applying ASURE to determine protective effect of Rhizobacteria under pesticide stress conditions

On comparing untreated, unchallenged healthy control with MCP stressed plant it can be understood that pesticide strongly affects plant growth. Of all the parameters studied, maximum deleterious impact seen on total plant biomass (35%) and total chlorophyll (65.13%). On the other hand, BI treatment affectively restored plant growth despite presence of pesticide stress resulting in maximum increase in total plant biomass (130%) and total chlorophyll (292%). Earlier studies too have shown negative correlation between plant growth vs. organic pollutant stress and positive impact of PGPR treatment [6,20,21]. During in-vitro pesticide degradation studies, risk of exposure to harmful pesticides exists while working with high dosages of pesticides involving, large number of treatments.

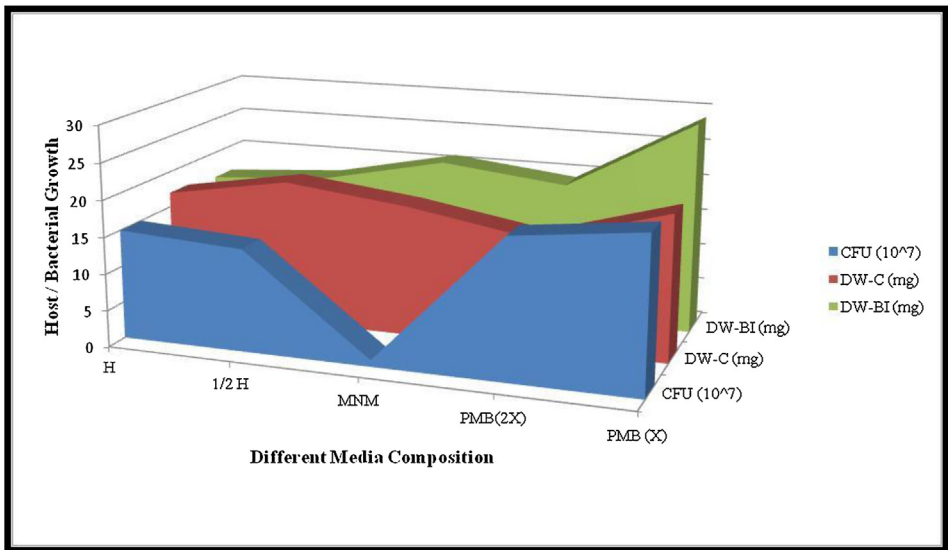


Fig. 7. Graph showing effect of different media composition on total plant biomass and BI.

Table 4

Impact of FI treatment on Plant growth.

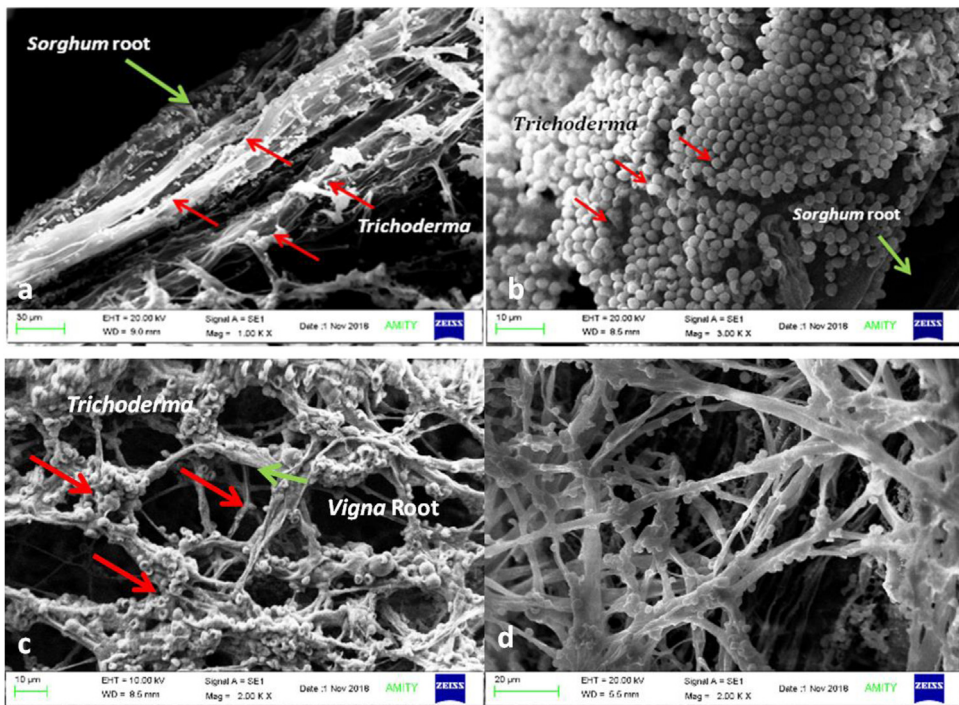
Parameters	Control	FI
Shoot length (cm)	5.90 ± 0.12	6.36 ± 0.124
Root length (cm)	4.05 ± 0.09	5.86 ± 0.04
Dry weight (mg)	19.0 ± 0.10	19.1 ± 0.14

By applying ASURE the exposure risk can be reduced manifold. This is amongst most beneficial aspects of proposed system “ASURE” when working with toxic pollutants including pesticides.

Applying ASURE for PGP studies with fungal inoculum

ASURE setup was further applied to observe growth promoting effect of fungal inoculum (FI) on host plants. This attempt was made to check the feasibility of utilizing BI optimised ASURE setup for FI based studies. Fungal isolate used in the study was a laboratory strain of *Trichoderma* species.

FI treatment (12th day) has positively impacted *Sorghum* showing 44.69% ↑ in root growth and 7.8% ↑ in shoot length (Table 4) as compared to un-inoculated control. FI favored development of secondary and tertiary roots (increased branching and profuse root architecture similar to BI treatment in Fig. 5). Though dry weight of FI treated plants is higher than untreated control, the difference was not statistically significant. Fig. 4(d) depicts FI treated host plant from ASURE setup. Enhanced root branching due to PGP treatment was also observed in earlier studies and attributed to biostimulation by fungal strains [14,16,22,23].



SEM images of FI association with hosts *Sorghum* (a, b) and *Vigna* (c, d) roots at different magnification

Fig. 8. SEM micrograph showing association between the host (*Sorghum* and *Vigna*) and fungi (FI).

Fig. 8(a, b) provides a closer view of FI treated host roots (Sorghum). Image proclaims aggressive colonization of fungus by 12th day, reflecting heavy sporulation all along the roots. In similar lines ASURE was further applied to another host plant (Vigna) along with same fungal inoculum. Fig. 8(c, d) is a loud display of strong association between Trichoderma and roots of Vigna. The experimental results provide ample confidence to claim that ASURE can be used with a wide choice of variables/treatments, multiple hosts and different microbes.

Acknowledgements

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.mex.2019.09.037>.

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