Life Science Informatics Publications

Research Journal of Life Sciences, Bioinformatics, Pharmaceutical and Chemical Sciences

Journal Home page http://www.rjlbpcs.com/

RJLBPCS

ISSN 2454-6348

Original Research Article **DOI - 10.26479/2016.0203.06**

EFFECT OF BIO-FERTILIZERS APPLICATION ON QUALITATIVE, QUANTITATIVE YIELD OF PHYTOCHEMICALS IN THREE DIVERGENT GROUPS OF PLANTS AND THEIR ANTIOXIDANT ACTIVITIES

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ABSTRACT: Studies on elicitation response of secondary metabolites such as alkaloids, tannins, flavonoids, lignans, phenols, anthocyanins, coumarins, terpenes etc., in the leaves of *Ocimum sanctum* (medicinal plant), *Phaseolus aureus* (pulses) and *Spinacia oleracea* (leafy vegetable) grown in different commercially available bio-fertilizers - Annapurna, Navajeevan with microbial inoculants was undertaken. There is a remarkable influence of bio-fertilizers on physical and chemical characteristics of plants such as seedling quality, germination percentage, number of leaflets, root, shoot length, total leaf area and biochemical constituents. Further quantitative and qualitative analysis of phytochemical constituents and the resultant antioxidant activities of the secondary metabolites in the plant extract are carried out. All the parameters are found to be enhanced in plants grown in Annapurna Bio-fertilizer when compared to Navajeevan and control. Knowledge of the identity and relative amounts of the secondary metabolites produced by plants is of great importance to several fields of basic and applied research in biology, chemistry and many other disciplines. Maintaining food production for the growing world population requires using new technology and intensifying production and management to grow more food on current cropland. Fertilizer is essential for accomplishing this.

KEYWORDS: Antioxidants, bio-fertilizers, phytochemical constituents, secondary metabolites

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1.INTRODUCTION

A survey of U.S. crop production estimated that there was an average of 40 percent decline of yields in corn crop without nitrogen (N) fertilizer. Depletion of other macronutrients like phosphorus (P) and potassium (K) exhibited even greater decline. Numerous studies have also confirmed the contributions of fertilizer to sustaining crop yields (Mikkelsen, 2008). Not only the quantitative yield, the quality of plant products is essential to produce nutritious food. The application of elicitors and bio-fertilizers has been well thought-out measure to improve the synthesis of secondary metabolites in medicinal plants. Allelochemicals are unique sources for pharmaceuticals, food additives, flavours and other industrial materials (Patel and Krishnamurthy, 2013). Production of secondary metabolites is influenced by different abiotic factors (temperature, UV radiation, salt conditions, metals, salinity, drought, flood and oxidative factors), biotic factors (pathogens, herbivores), soil conditions, soil nitrogen and antioxidants (Zhu, 2002). Bio-fertilizers are substances which contain living microorganisms, when applied to seed, plant surfaces or soil, colonizes the Rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant. Bio-fertilizers add nutrients through the natural processes of nitrogen fixation, solubilizing phosphorus, and stimulating plant growth through the synthesis of growth-promoting substances. The microorganisms in bio-fertilizers restore the soil's natural nutrient cycle and build soil organic matter (Kapoor et al., 2015). Use of bio-fertilizers ensures healthy plants growth, while enhancing the sustainability and the vigor of the soil. A preferred scientific term for beneficial bacteria which perform different functions is "plant-growth promoting rhizobacteria" (PGPR). They are extremely advantageous in enriching soil fertility and fulfilling plant nutrient requirements by supplying the organic nutrients through microorganisms and their products and do not contain any chemicals which are harmful to the living soil (Vessey, 2003). Kalmegh, Ashwagandha, Tulsi, Azadirachta, Curcuma, Ocimum, green leafy vegetables etc. are important medicinal plants mentioned in ancient ayurvedic literature. These medicinal plants are formulated into various drugs that cure fever to acute jaundice and heart diseases to cancer. Plants are major source of micro nutrients and vitamins. They are low in fat, high in dietary fiber, rich in folic acid, vitamin C, potassium and magnesium, as well as contain bioactive compounds such as phenolic compounds, flavonoids, alkaloids, tannins, lignans, phenols, terpenes etc. a host of phytochemicals such as lutein, β-cryptoxanthin, zeaxanthin and β-carotene have been isolated and identified. Their anti-septic and other therapeutic activities have been reviewed by Joshi et al., and Srinivas Naik et al., (Joshi et al., 2011; Srinivas Naik et al., 2015). They are useful in improving immune function and reduce the risk of cancer and heart diseases. Essential oils extracted from the leaves of *Ocimum sanctum* L. have been found to inhibit growth of *E. coli, B. anthracis* and *P. aeruginosa* (Rahman et al., 2011). Tulsi has anti-tubercular activity and inhibits growth of *M. tuberculosis* and also possess anti-fungal and

anti-viral activity (Unander et al., 1990).The aqueous and ethanolic extract of *Spinacia oleracea* a green leafy vegetable showed potent antioxidant and anti diarrhoeal activity (Garg et al., 2010; Ko et al., 2014). Secondary metabolites act as antioxidants which are widely used in dietary supplements and have been investigated for the prevention of diseases such as cancer, coronary heart disease and even altitude sickness and also have many industrial applications as preservatives in foods and in cosmetics. The irregular and low germination was the main problem in the propagation of many medicinal plants (Rachel et al., 2015). Application of fertilizers such as chemical fertilizers increases the productivity and growth but decreases the soil conditions and also effect the human kind. The enormous and improper use of chemical fertilizers leads to fast deterioration of physical, chemical and biological properties of soil. To avoid the residual toxicity of chemical fertilizers, it is always advisable to use organic cultivation practices. Bio-fertilizers are eco-friendly and decrease these irregularities and enhance germination. They also reduce the chemical inputs and enhance the yield both qualitatively and quantitatively (Darzi, 2012; Kapoor et al., 2004). The use of bio-fertilizers has been reported to be beneficial for the cultivation of vegetable and cereals by many workers (Hadas and Okon, 1987).The active principles of bio-fertilizers depend on the total biomass yields which further depends on the climatic feature, method of organic agro-techniques, water management and also fertilizer applications. So yield improvement, soil structure improvement, physical properties and especially its water holding capacity can be achieved by standardizing the agronomy and its practices especially with respect to those parameters which could be the great asset in tropics and for its sustainability (Abiven et al., 2009). The use of organic manure in combination with bio-fertilizers offers a great opportunity to increase the crop production with less cost. Most of the bio-fertilizers contain microorganisms like *Rhizobium, Azotobacter, Azospirillum* and blue green algae (BGA). Bio-fertilizers are naturally occurring organic fertilizers which includes manure, slurry, worm castings, peat, seaweed, compost, humic acid, and guano etc. and also bio extracts such as Rhizobacteria, Azotobacter, Azospirullum, phosphate solubilizing bacteria, castor and Trichoderma. The present investigation was undertaken to study the effect of commercial bio-fertilizers on micronutrient content, morphological and biochemical parameters in three divergent groups of plants belonging to pulses, vegetable and medicinal categories and their resultant antioxidant activities.

2. MATERIALS AND METHODS

Three plants were selected to study the effect of bio-fertilizers on germination, growth and biochemical constituents. The research work consists of both physical and chemical characteristics of selected plants that are treated with bio-fertilizers.

Bio-fertilizers:

Annapurna and Navajeevan are two commercially available bio-fertilizers which are used in the present study and were purchased from Sri Satya Sai Agri Chemicals, Visakhapatnam.

Plants:

Three different plants were selected for the present study. Seeds were collected from Dr YSR Horticultural University, Tadepalligudem, Andhra Pradesh, India. Healthy seeds were chosen and used for experiments.

Scientific Name	Vernacular Name	Plant part used	Family
Ocimum sanctum	→Tulasi (Medicinal plant)	→ Leaves	→*Labitae*
Phaseolus aureus	→Green gram (Pulse plant)	→Leaves	→*Leguminoceae*
Spinacia oleracea	→Spinach (Leafy vegetable)	→Leaves	→*Amaranthaceae*

Soil amendments:

Soil was prepared by combining the selected commercial bio- fertilizers Annapurna and Navajeevan in the ratio of 1:1. Both the fertilizers were mixed 3days prior to sowing. All pots were irrigated with tap water and left for organic material decomposition.

Treatments and experimental design:

Three treatments with three replicates and three soil amendment systems are considered.

They are i) Soil without bio-fertilizer – Control

ii) Soil with Annapurna bio-fertilizer and

iii) Soil with Navajeevan bio-fertilizer.

Pots were arranged on a bench in a wire-netting greenhouse under natural environmental conditions.

Germination (%):

The number of seeds germinated in each bio-fertilizer was counted on 7^{th}day and the germination percentage was calculated by using the following formula

$$\text{Germination \%} = \frac{\text{No. of seeds germinated}}{\text{Total no. of seeds sown}} \times 100$$

Leaflet Number

The number of leaf lets is counted in the respective plants. Then bio-fertilizer treated plants are compared with control i.e. plants treated with soil. The number of leaflets were counted on 7^{th}day (Saravanan et al., 2014).

Seedling quality parameters: The morphological parameters of the plants were analyzed at 5 days intervals, three plants were collected from each category and they were analyzed for their morphological parameters such as shoot length, root length, total leaf area (Saravanan et al., 2014).

Shoot and Root length:

The influence of the bio-fertilizers on the selected plants were analyzed by measuring the root length and shoot length of plants using centimeter scale and recorded. The seedlings were uprooted carefully

and growth of the root system is considered without any destruction. The shoot length was measured from base of the stem to the last leaf open and the root length was measured from base of the main stem to end of the root by using scale (centimeter).

Total leaf area:

The plant samples were collected periodically (1, 15, 30, 45 and 60days) and the length and breadth of the leaf samples were measured as described by Yoshida *et al.*, 1976 (James H Cock, 1976; Rameshkumar et al., 2013; Seshadri et al., 2015). The total leaf area was calculated by multiplying the length and breadth with Kemp's constant.

Leaf area (cm2) = K × length × breadth

Where, K = Kemp's constant (for dicot leaves = 0.66)

Processing of plant samples:

The leaves from the selected plants were properly washed with tap water and then rinsed with distilled water. The rinsed leaves were dried in an oven at 35 – 40^{o}C for 3 days. The dried leaves of each plant were pulverized using a mortar and pestle, to obtain powdered form. And the powder was stored in airtight glass containers, protected from sunlight then it was used for further studies.

Preparation of plant sample:

10g of pulverized leaves were soaked separately in 100 ml of each of distilled water, ethanol and methanol for 72hrs. The soaked material was stirred using a sterile glass rod with certain time intervals and the extracts were filtered using filter paper. Then, the extracts were concentrated to half of the original extracts and stored in airtight container and are used for the further analysis.

Phytochemical Screening Methods

Several biochemical constituents were evaluated qualitatively and quantitatively using standard protocols (Geetha and Geetha, 2014; Sadiq, 2014).

Qualitative analysis:

The presence of various biochemical constituents such as tannins, saponins, glycosides, flavonoids, terpenoids, coumarins, alkaloids (Agnel Arul John and Shobana, 2012; Firdouse and Alam, 2011) phlobatannins, anthocyanins (Edeoga et al., 2005) and sterols (Durairaj et al., 2014) were assessed at different time intervals i.e. on 30th and 60th day old seedlings of three selected plants that are grown in three different conditions.

Quantitative Analysis:

The amount of phytochemical constituents was estimated according to the standard protocols. Determination of tannins by Van Buren and Robinson (Edeoga et al., 2005; Van Buren and Robinson, 1969); saponins by Obadoni and Ochuko (Obadoni and Ochuko, 2002); flavanoids by Bohm and Koupai-Abyazani (Bohm and Koupai-Abyazani, 1994); alkaloids by Richardson and Harborne (Richardson and Harborne, 1985) and phenols by Edeoga et al., (Edeoga et al., 2005).

Determination of Total phenolic content:

The total phenolic content was determined by the Folin Cio-calteu (FC) reagent method (Lallianrawna et al., 2013; Siddique et al., 2010; Singleton et al., 1965). When phenols react with phosphomolybdic acid in Folin Cio-calteau reagent in alkaline medium it gives Molybdenum blue coloured complex. Based on this principle, to 100µg of plant extract, 0.5 ml of distilled water, 2.0ml Folin Cio-calteau reagent and 2.0ml 7.5% Na_2CO_3 were added. The mixture was allowed to stand for 15 min at 45^oC, shades of blue colour was observed and the absorbance was measured at 765 nm using spectrophotometer. Total phenolics levels were expressed as Gallic acid equivalents (mg g –1 of dry mass).

Determination of Antioxidant activity:

1. **1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay**

   The ability of the extracts to scavenge DPPH radical was assessed spectrophotometrically by the method of Gyamfi et al., (Gyamfi et al., 1999). To 5 ml of methanolic DPPH, 50µl of plant extract was added. The reaction mixture was incubated at 37^oC for 30min in dark and the absorbance was read at 517nm using spectrophotometer. BHT (Butylated hydroxyl toluene) was used as standard.

2. **Iron (III) to Iron (II) reduction assay**

   The reductive capacity of the extracts was assessed spectrophotometrically as described by Yildirim et al.,(Yildirim et al., 2000). In the presence of antioxidants, ferric is reduced to ferrous state which forms a Perl's Prussian blue coloured complex and the absorbance read at 517nm.

3. **Superoxide anion scavenging activity**

   Superoxide anion scavenging activity of the extracts was validated according to the method of Liu et al.,(Liu et al., 1997). Superoxide anions were generated in a non-enzymatic PMS-NADH system by the oxidation of NADH and assayed by reduction of NBT (Nitro Blue Tetrazolium) and absorbance read at 560nm.

4. **Enzyme Assays**

   Estimation of Catalase

   The amount of catalase was determined by the standard method of Maehly and Chance (Maehly and Chance, 1954). An aliquot of 1ml of the supernatant of the enzyme extract was added to the reaction mixture containing 1ml of 0.01MH_2O_2 and 3ml 0.1 M phosphate buffer. It was incubated for 5mins at 20^oC then the reaction was stopped by adding 10ml of 1% H_2SO_4. The extracts were titrated against 0.005N $KMNO_4$ and the catalase activity was expressed at n moles of H_2O_2 utilized (units' minutes^{-1}mg^{-1} protein).

Polyphenol Oxidase (PPO)

Polyphenol oxidase activity was assessed by the method of Kumar and Khan (Kumar and Khan, 1982). Assay mixture for PPO contained 2 ml of 0.1 M phosphate buffer (pH 6.0), 1 ml of 0.1 M catechol and 0.5 ml of enzyme extract. This mixture was incubated at 25^{o}C for 5 min, then the reaction was stopped by adding 1 ml of 2.5 N H_2SO_4. The absorbance was read at 495 nm. PPO activity was expressed in U mg^{-1} protein (U=change in 0.1 absorbance $min^{-1}mg^{-1}$ protein).

Peroxidase activity (POX)

Peroxidase activity was determined spectrophotometrically at 250nm with a UV -visible spectrophotometer using guaiacol as the substrate and H_2O_2 as the hydrogen donor described by Pine et al.,(Pine et al., 1984). To 25 µl of the extract to 2 ml of a solution containing 50 mM potassium phosphate buffer pH 6.8, 20 mM guaiacol and 20 mM H_2O_2 were added. After incubation at 30°C for 10 min, the reaction was stopped by adding 0.5 ml 5% (v/v) H_2SO_4 and the absorbance was read at 480 nm. One POX unit was defined as U mg^{-1} protein (U=change in 0.1 absorbance min^{-1} mg^{-1} protein).

5. Estimation of Nitrogen using Nitrogen Analyzer

Total N in plants is estimated by Kjeldahl method (Magomya et al., 2014). In plants, N is present in protein form and digestion of the sample with H_2SO_4 containing digestion mixture (10 parts potassium sulphate and 1-part copper sulphate) is required for estimation. Sample size may be 0.5–1.0 g depending on the type of crop and the plant part. The procedure involves sample digestion, distillation and estimation of Nitrogen.

4. RESULTS AND DISCUSSION

Bio-fertilizers treatment/ organic agro techniques have a great effect on all the growth stages of crop plants. The seed inoculations with bio-fertilizers like soil and commercially available Annapurna and Navajeevan bio-fertilizers at various treatments has significantly increased the plant growth, secondary metabolites and antioxidant properties of *Ocimum sanctum* (Tulasi), *Phaseolus aureus* (Green gram) and *Spinacia oleracea* (Spinach) plants.

Influence of bio-fertilizers on seedling quality parameters:

A significant variation in plant height and number of leaves due to application of bio-fertilizers was found. Statistically, significant increase in the plant height, number of leaflets, Shoot length, Root length and leaf area was observed. The morphological parameters of selected plants were analyzed at 5 days' interval gaps. The three plants were collected from each soil amendments under study.

Germination (%) and Leaflets:

In the present study, plants that are treated with bio-fertilizers showed higher germination percentage compared to control. The number of seeds germinated in each bio-fertilizer was counted on 7th day and the germination percentage was calculated. The maximum, seed germination was observed in

Spinacea oleracea with 80% in Annapurna bio-fertilizer. Germination in both Navajeevan bio-fertilizer and control (70%) were equal. *Phaseolus aureus seeds exhibited 70% germination* in both Annapurna and Navajeevan, 60% was observed in control. *Ocimum sanctum* showed 60% germination in Annapurna and Navajeevan bio-fertilizers and 50% in control (Table – 1). Enhancement of seed germination might be attributed to the role of rhizobacteria, *Azospirillum* and phosphobacteria in enhancing the availability of nitrogen and phosphorus in the soil thus making these nutrients available to the germinating seed with consequent enhancement in the metabolic activity resulting in higher germination (Rajasekaran et al., 2015). The opening of the cotyledons exposes the shoot apical meristem and the plumule consisting of the first true leaves of the young plant. The seedlings sense light through the light receptors phytochrome and cryptochrome. Mutations in these photo receptors and their signal transduction components lead to seedling development that is at odds with light conditions So, number leaflets were considered in the present study. The number of leaflets was counted on 7th day. The maximum, number of leaflets were counted in *Ocimum sanctum* with 4 no's followed by *Phaseolus aureus* with 3 no's and *Spinacea oleracea* with 1 no's. The selected plants equally produced number of leaflets in all the three conditions (Soil, Annapurna and Navajeevan bio-fertilizers).

Table – 1: Percentage of seed Germination and number of Leaflets

S. No.	Name of the Plant	% of Seed Germination			No. of Leaflets		
		Control soil	Annapurna	Navajeevan	Control soil	Annapurna	Navajeevan
1.	*Ocimum sanctum*	50%	60%	60%	4	4	4
2.	*Spinaceaoleracea*	70%	80%	70%	1	1	1
3.	*Phaseolus aureus*	60%	70%	70%	3	3	3

Shoot and Root length:

The influence of bio-fertilizers on shoot and root length of selected plants at various stages (1, 15, 30, 45 and 60) of its growth was represented in table – 2. The plants produced elongated roots and shoots on bio-fertilizer treatment. Maximum length of roots was observed in *O. sanctum* followed by *P. aureus* and *S. oleraceae* and increased shoot length was observed in *S. oleraceae* followed by *O. sanctum* and *P. aureus*. Plants that are treated with Annapurna bio-fertilizers showed increased growth compared with Navajeevan bio-fertilizers and control soil. The increase in growth attributes to microorganisms that are present in bio-fertilizers, which stimulates the plant growth by suppling nutrients by their colonization at the rhizosphere or by their symbiotic association. The association

also regulates the physiological process in the ecosystem, by the involvement of organic matter decomposition and atmospheric nitrogen fixation (Rajasekaran et al., 2015).

Parameters		Days	*Ocimum sanctum*			*Spinacea oleracea*			*Phaseolus aureus*		
			Soil	Annapurna	Navajeevan	Soil	Annapurna	Navajeevan	Soil	Annapurna	Navajeevan
Length (cm)	Root	1	0	0	0	0	0	0	0	0	0
		15	2.5	2.8	2.8	1.2	1.5	1.5	1.5	1.9	1.7
		30	5.1	5.7	5.5	1.5	1.8	1.7	2.2	2.2	2.3
		45	7.2	7.6	7.1	2	2.1	2.1	5.7	6.3	7
		60	8.5	9	8.5	2.5	2.9	2.7	8	8.2	8.3
	Shoot	1	0	0	0	0	0	0	0	0	0
		15	3.1	5.2	3.5	4	5.3	4	1.8	3	2
		30	8.2	8.5	8.2	8.9	9	8.4	6.2	8.5	6.5
		45	15	16	15.7	15.6	17	17	12.2	15	13
		60	23.5	28	25	25	31	28	20	25	21
Leaf area (cm^2)		1	0	0	0	0	0	0	0	0	0
		15	0	0	0	1.32	3.66	3.66	0.99	1.32	1.18
		30	0.99	1.98	1.32	10.56	19.8	16.5	2.97	3.15	2.64
		45	1.32	3.96	2.64	27.72	36.96	32.34	3.3	4.35	3.96
		60	4.62	5.28	4.62	36.96	52.8	42.52	4.62	5.77	4.94

Leaf Area:

Table 2: Root, Shoot length and Leaf area measured at time interval of 15 days upto 60days

The influence of bio-fertilizers on total leaf area of *S. oleraceae*, *O. sanctum* and *P. aureus* at various stages of its growth was reported in table – 2. The highest total leaf area was recorded in *S. oleraceae* followed by *P. aureus.* Similarly, the lowest total leaf area was recorded in *O. sanctum* treated with bio-fertilizer at 1, 15, 30, 45 and 60days respectively. Plants grown in Annapurna bio-fertilizer exhibited maximum increase than the Navajeevan and control soil. The increase of leaf area is attributed to the nitrogen that was made available in the soil due to the organism present in the bio-fertilizers. Nitrogen application increases the metabolism rate and transport of growth promoters in the plants which results in promoting length of the leaf and leaf area (Ahmad et al., 2011).

Qualitative analysis of phytochemical constituents:

The analysis of phytochemical constituents in plants is essential for the extraction, isolation, identification and purification of various metabolites with medicinal value. Table – 3 represents qualitative analysis of phytochemical constituents that were identified in three different plants namely *Ocimum sanctum, Spinacia oleraceae* and *Phaseolus aureus* grown in three different foliar treatments

(Control soil, Annapurna and Navajeevan bio-fertilizers) at 30 and 60days respectively. *O. sanctum* showed the presence of tanins, phlobatannins, flavanoids, terpenoids, glycosides, alkaloids, steroids, anthocyanins, coumarins and phenols at both 30 and 60days. *P. aureus* at 30 and 60days has shown the presence of tanins, phlobatannins, flavanoids, glycosides, alkaloids, steroids, anthocyanins and phenols respectively. At 30days *S. oleraceae* showed tanins, phlobatannins, saponins, flavanoids, terpenoids, glycosides, alkaloids, steroids, coumarins and phenols and at 60days tanins, phlobatannins, flavanoids, glycosides, alkaloids, steroids, anthocyanins and phenols. Saponins were completely absent in *Ocimum sanctum*, at both 30 and 60 days. In *Spinacia oleraceae,* at 30days Anthocyanins and at 60days saponins, terpenoids and coumarins were absent and in *Phaseolus aureus*, saponins, terpenoids and coumarins were absent in both samples of different time intervals.

Table – 3: Phytochemical analysis of selected plants in different soil treatments

Plant	Phytochemical	30 Days			60 Days		
		Control	Annapurna	Navajeevan	Control	Annapurna	Navajeevan
Ocimum sanctum	Tanin	+	+	+	+	+	+
	Phlobatannins	+	+	+	+	+	+
	Saponin	-	-	-	-	-	-
	Flavanoids	+	+	+	+	+	+
	Terpenoids	+	+	+	+	+	+
	Glycosides	+	+	+	+	+	+
	Alkaloids	+	+	+	+	+	+
	Steroids	+	+	+	+	+	+
	Anthocyanins	+	+	+	+	+	+
	Coumarins	+	+	+	+	+	+
	Phenols	+	+	+	+	+	+
Phaseolus aureus	Tanin	+	+	+	+	+	+
	Phlobatannins	+	+	+	+	+	+
	Saponin	-	-	-	-	-	-
	Flavanoids	+	+	+	+	+	+
	Terpenoids	-	-	-	-	-	-
	Glycosides	+	+	+	+	+	+
	Alkaloids	+	+	+	+	+	+
	Steroids	+	+	+	+	+	+
	Anthocyanins	+	+	+	+	+	+
	Coumarins	-	-	-	-	-	-
	Phenols	+	+	+	+	+	+

Spinacia oleracea	Tanin	+	+	+	+	+	+
	Phlobatannins	+	+	+	+	+	+
	Saponin	+	+	+	-	-	-
	Flavanoids	+	+	+	+	+	+
	Terpenoids	+	+	+	-	-	-
	Glycosides	+	+	+	+	+	+
	Alkaloids	+	+	+	+	+	+
	Steroids	+	+	+	+	+	+
	Anthocyanins	-	-	-	+	+	+
	Coumarins	+	+	+	-	-	-
	Phenols	+	+	+	+	+	+

Quantitative analysis of phytochemical constituents:

Quantitative determination of percentage of biochemical constituents was carried out in the selected three plants. Tanins, saponins, flavonoids, terpenoids, alkaloids, steroids, anthocyanins, coumarins and phenols were present in the selected plants at 30 and 60days which are inoculated with bio-fertilizers and soil.In *Ocimum sanctum*, the highest amount of the phytochemical synthesized by plant was terpenoids followed by anthocyanin, coumarins, phenols and traces of tanins, flavonoids, alkaloids and steroids. Where as in *Phaseolus aureus*, anthocyanin is majorly synthesized by the plant followed by tanins and phenols and traces of alkaloids, flavonoids and steroids. Similarly, terpenoids are highly synthesized by *Spinacia oleracea* followed by tanins, phenols, saponis and traces of alkaloids, flavonoids and steroids (Table – 4). Of the three treatments, plants grown in Annapurna bio-fertilizer exhibited highest amount of secondary metabolites quantitatively (Figure –1to 3).

Figure – 1: Quantitative analysis of phytochemicals in *O. sanctum*

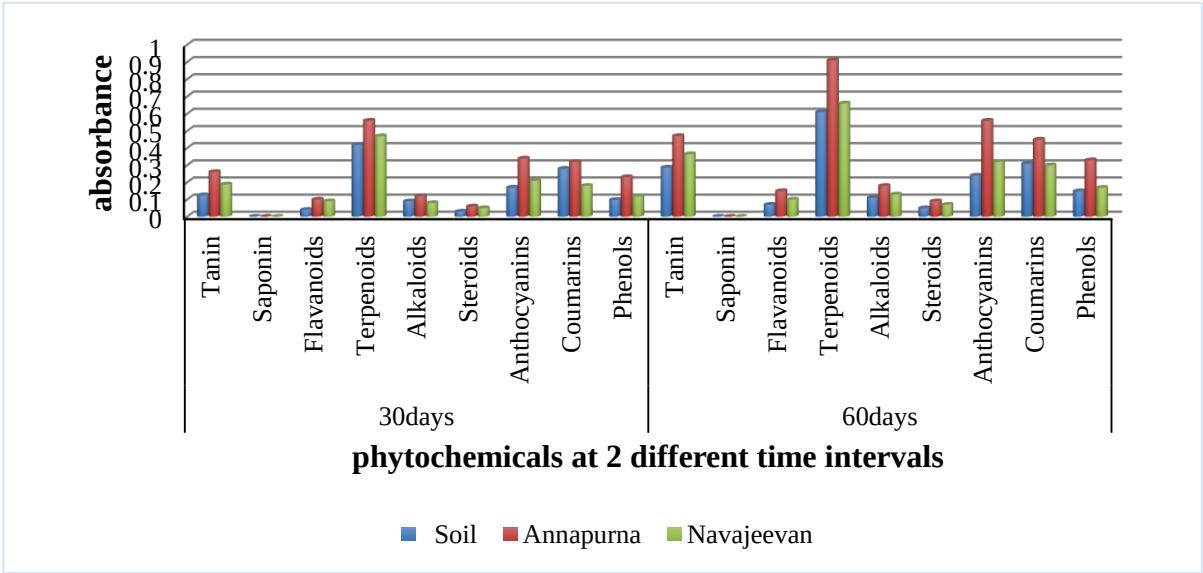


Figure – 2: Quantitative analysis of phytochemicals in *P. aureus*

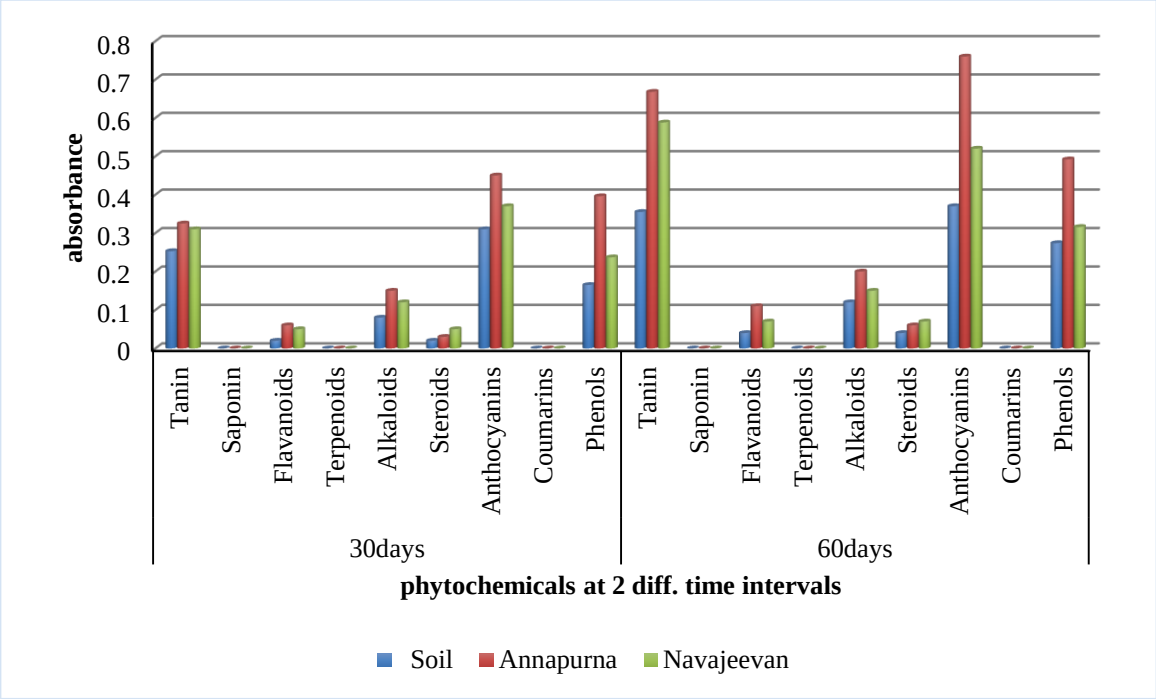
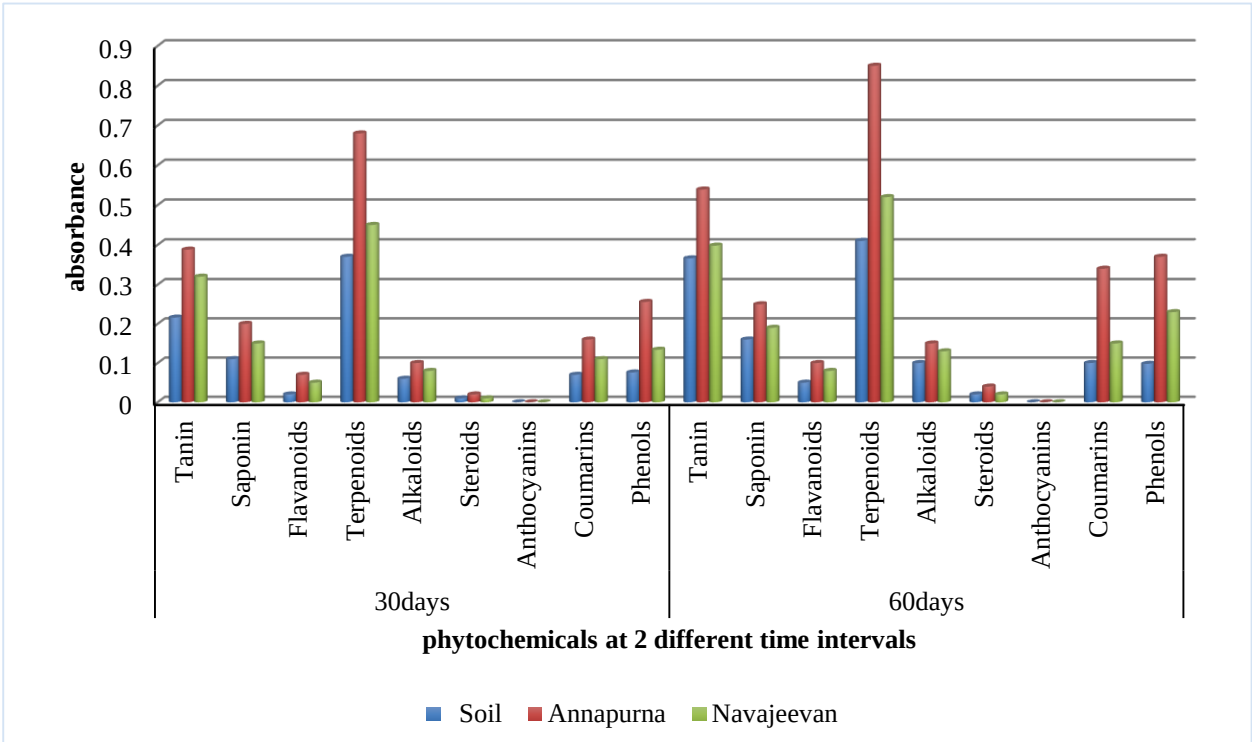


Figure – 3: Quantitative analysis of phytochemicals in *S. oleraceae*



Peer review under responsibility of Life Science Informatics Publications
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The findings of this study reveal that, all the microbial strain combinations in Annapurna bio-fertilizer either individually or in consortia showed enhanced pattern of vegetative growth of plants at various stages in pot experiment under natural environmental condition. Microbial inoculants consisting of living cells like Azospirillum, Azatobacter, PSB, Trichoderma present in the bio-fertilizer are found to enhance the parameters under study. Whereas Navajeevan which is commercially sold as bio-fertilizer is inferior to Annapurna owing to its organic nature rather than biological. Navajeevan though has many ingredients like bio extracts, organic fertilizers: manure, compost, worm castings, peat, sea weed extracts, humic acid, guano and sea weed amino acids the parameters under study were not significantly enhanced. This may be due to lack of sustained release of nutrients to supply the required elements in a readily available form for plant use.

Table – 4: Quantitative analysis of phytochemical constituents

Plant	Phytochemical	30 Days			60 Days		
		Control	Annapurna	Navajeevan	Control	Annapurna	Navajeevan
Ocimum sanctum	Tanin	0.125	0.261	0.189	0.287	0.471	0.365
	Saponin	0	0	0	0	0	0
	Flavanoids	0.04	0.1	0.09	0.07	0.15	0.1
	Terpenoids	0.42	0.56	0.47	0.61	0.91	0.66
	Alkaloids	0.09	0.12	0.08	0.11	0.18	0.13
	Steroids	0.03	0.06	0.05	0.05	0.09	0.07
	Anthocyanins	0.17	0.34	0.21	0.24	0.56	0.32
	Coumarins	0.28	0.32	0.18	0.31	0.45	0.3
	Phenols	0.098	0.231	0.118	0.149	0.33	0.169
Phaseolus aureus	Tanin	0.253	0.325	0.31	0.355	0.668	0.588
	Saponin	0	0	0	0	0	0
	Flavanoids	0.02	0.06	0.05	0.04	0.11	0.07
	Terpenoids	0	0	0	0	0	0
	Alkaloids	0.08	0.15	0.12	0.12	0.2	0.15
	Steroids	0.02	0.03	0.05	0.04	0.06	0.07
	Anthocyanins	0.31	0.45	0.37	0.37	0.76	0.52
	Coumarins	0	0	0	0	0	0
	Phenols	0.165	0.396	0.237	0.274	0.492	0.316
Spinaci	Tanin	0.216	0.388	0.32	0.366	0.539	0.398
	Saponin	0.11	0.2	0.15	0.16	0.25	0.19

	Flavanoids	0.02	0.07	0.05	0.05	0.1	0.08
	Terpenoids	0.37	0.68	0.45	0.41	0.85	0.52
	Alkaloids	0.06	0.1	0.08	0.1	0.15	0.13
	Steroids	0.01	0.02	0.01	0.02	0.04	0.02
	Anthocyanins	0	0	0	0	0	0
	Coumarins	0.07	0.16	0.11	0.1	0.34	0.15
	Phenols	0.076	0.256	0.134	0.098	0.37	0.23

Determination of Antioxidant Activity

Antioxidants play an important role in human health as they are capable of inhibiting the free radical damage at very low concentrations. In 1990s, scientist made a theory that people who consume less antioxidant foods in their diet are more prone to chronic disorders. This made many scientists to investigate the antioxidant rich plants and use them as nutraceuticals or as a food supplement. In this study the antioxidant levels were determined in the selected three plants in different conditions and at two different time intervals i.e. after 30 days and 60days of plant growth. Total phenols, DPPH radical scavenging activity, reduction of Fe^{3+} to Fe^{2+} ions, superoxide dismutase test and enzyme activity assays namely catalase, peroxide and polyphenol oxidase were done to determine the antioxidant potential of the plants. Among the three groups of plants highest amount of antioxidant activity were observed in *Ocimum sanctum* a medicinal plant. Total antioxidant activity was highest followed by catalase, DPPH, reduction of Fe^{3+} to Fe^{2+}ions, polyphenols and little of superoxides, phenols and peroxides (Table– 5). In *Phaseolus aureus* catalase activity was highest followed by DPPH, reduction of Fe^{3+} to Fe^{2+} ions, peroxide and little of phenols, polyphenols and superoxide. Similarly, the antioxidant activity levels of *Spinacia oleracea* coincides with the activities of *P. aureus*. Interestingly, plants that are grown in Annapurna bio-fertilizer showed a maximum antioxidant activity followed by Navajeevan bio-fertilizers and then Normal soil (Figure – 4 to 6). In all the three plants, catalase activity was highest in all three conditions.

Table – 5: Determination of antioxidant activity

Plant	Phytochemical	30 Days			60 Days		
		Control	Annapurna	Navajeevan	Control	Annapurna	Navajeevan
Ocimum sanctum	Total phenols	0.157	0.343	0.295	0.209	0.455	0.265
	DPPH	0.762	0.944	0.873	0.995	1.065	1.037
	Fe3+toFe2+	0.239	0.405	0.321	0.432	1.436	0.539
	Superoxide	0.138	0.34	0.279	0.429	0.632	0.487
	Catalase	2	2.5	2.2	2.5	2.9	2.6
	Peroxide	0.094	0.246	0.148	0.17	0.393	0.261
	Polyphenols	0.328	0.561	0.402	0.497	0.752	0.623
Phaseolus aureus	Total phenols	0.178	0.308	0.412	0.228	0.434	0.552
	DPPH	0.649	0.967	0.684	0.789	1.103	0.762
	Fe3+toFe2+	0.366	0.434	0.365	0.802	0.98	0.915
	Superoxide	0.158	0.249	0.253	0.21	0.327	0.305
	Catalase	1	1.8	1.5	1.3	2.4	1.7
	Peroxide	0.482	0.63	0.613	0.786	0.897	0.815
	Polyphenols	0.174	0.218	0.254	0.209	0.314	0.25
Spinaciaoleracea	Total phenols	0.185	0.267	0.215	0.263	0.361	0.275
	DPPH	0.963	1.045	1.012	1.01	1.232	1.115
	Fe3+toFe2+	0.167	0.256	0.218	0.551	0.621	0.739
	Superoxide	0.245	0.395	0.317	0.322	0.592	0.415
	Catalase	1.1	1.6	1.7	1.3	1.9	1.8
	Peroxide	0.539	0.684	0.583	0.754	0.899	0.856
	Polyphenols	0.149	0.33	0.242	0.206	0.476	0.369

Figure – 4: Determination of antioxidant activity in *Ocimum sanctum*

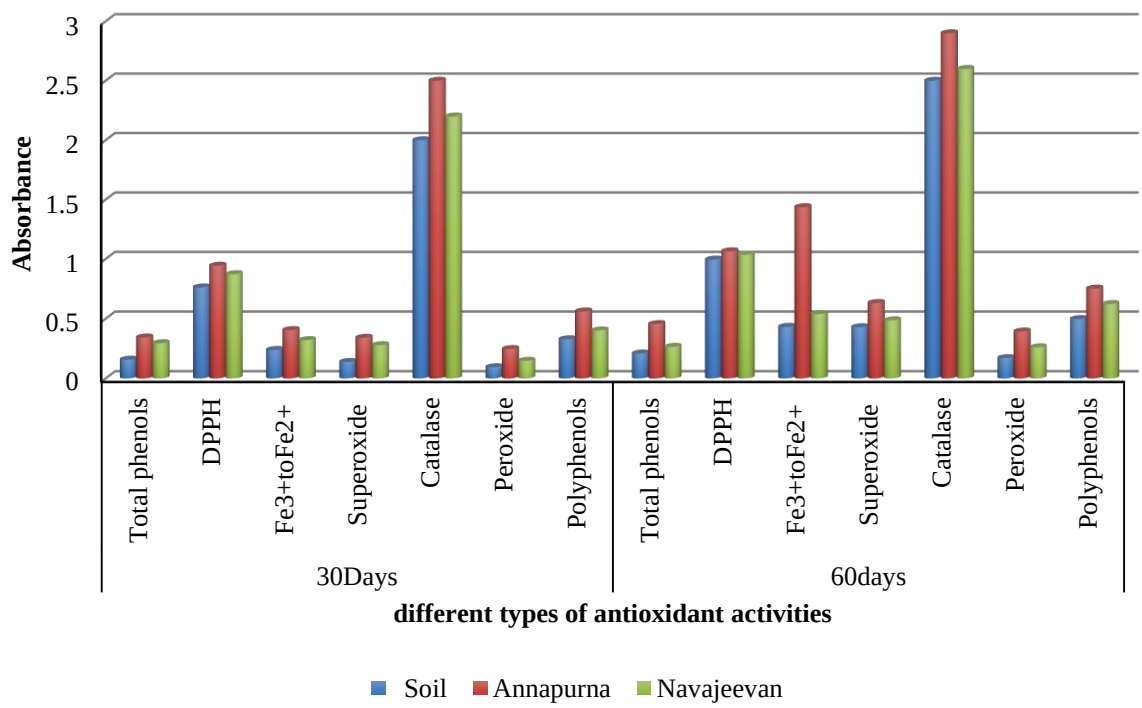


Figure – 5: Determination of antioxidant activity in *Phaseolus aureus*

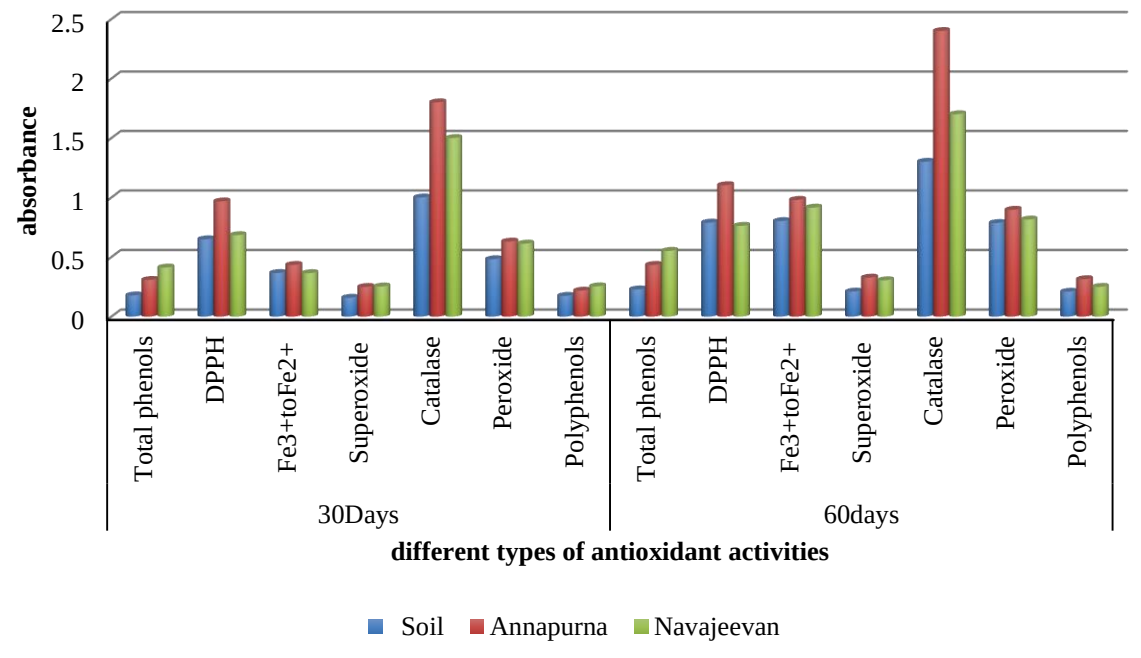
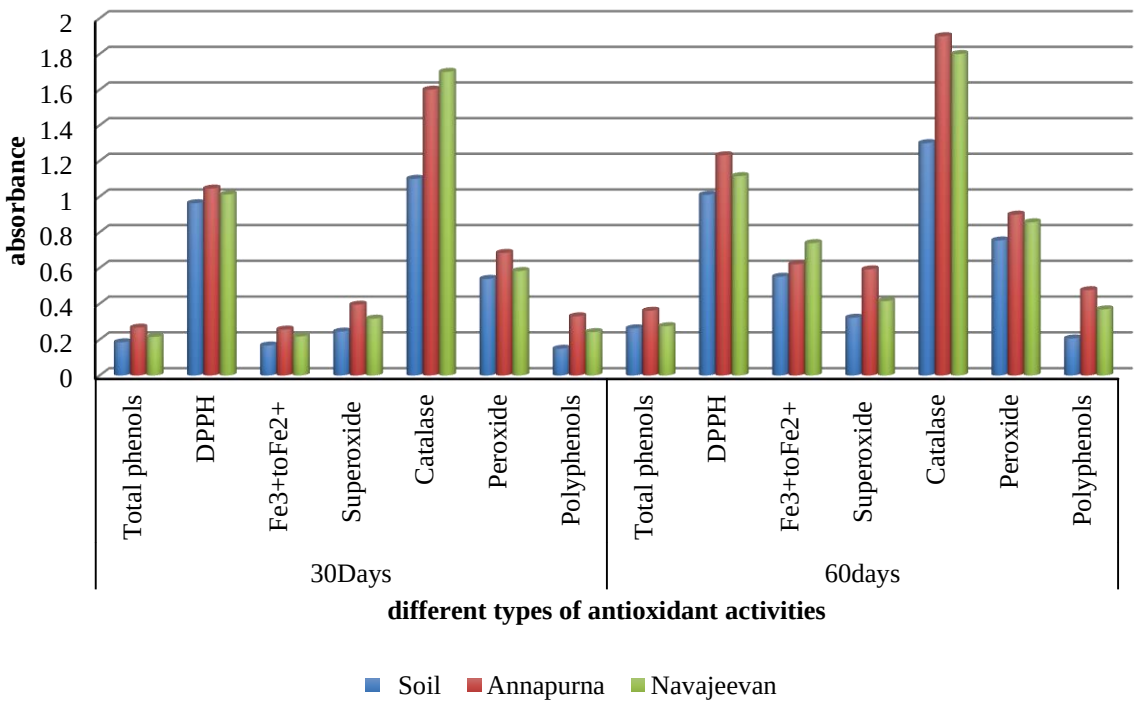


Figure – 6: Determination of antioxidant activity in *Spinaciaoleracea*



4. CONCLUSION

This study has revealed that there is a huge potential for the use of bio-fertilizers in a wide variety of crop plants. Application of bio-fertilizers promoted healthy growth of plants while enhancing sustainability of soil. A higher secondary metabolite production augmented antioxidant activities. Apart from this there was an enhancement in the morphological parameters also. Plants with antioxidant activities have been reported to possess free radical scavenging activity. Free radicals are known as major contributors to several clinical disorders such as Diabetes mellitus, cancer, liver diseases, renal failure and degenerative diseases. All the three plants showed significant antioxidant activities in all three conditions, comparatively plants that are grown in Annapurna bio-fertilizers showed highest activity. The enhanced expression will have beneficial impact in production of pharmaceutical and nutraceutical products of commercial importance. Environmental stewardship and proper use of fertilizer products plays a critical role in meeting global food demand and ensuring increased food production in a responsible and sustainable manner

CONFLICT OF INTEREST

The authors declare that no competing financial interests exist.

ACKNOWLEDGMENTS

Authors are thankful to Department of Biochemistry and Bioinformatics, Institute of Science, GITAM University, Visakhapatnam, for providing laboratory facilities to carry out the present work.

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