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ORIGINAL RESEARCH

Evaluation of lignin waste as potential carriers for phosphate solubilizing bio-fertilizers: A zero waste technology

Gayathri Unnikrishnan[1*], Vijayaraghavan Ramasamy[2]

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Abstract

Purpose Incineration of plant biomass and refusing lignin rich effluent from paper industry to water bodies were seen as usual practice. This product-oriented research had a promising solution for recycling agro waste. Likewise, high quality handmade papers from dead-dry leaves of *Ficus citrifolia*, *Swietenia mahagoni*, *Pinus roxburgii* and *Musa acuminate* were obtained. Lignin was found to improve soil fertility and nutrient reservoir for microbial growth. So, this research managed and utilized lignin as liquid carriers to phosphate solubilizing bio-fertilizers (PSBs)- *Meyerozyma gullerimondi* and *Providencia rettgeri*.

Method Handmade papers made were checked for quality by determining breaking length, burst factor, and gram per square meter (GSM). The essential nutrients in lignin waste were analyzed using FESEM-EDX (Field emission scanning electron microscope- energy dispersive X-ray spectroscopy). This confirmed suitability of lignin as fermenting carrier media for phosphate solubilizing biofertilizer (PSBs). After fermentation, nutrients were quantified using Carbon Hydrogen Nitrogen Sulphur (CHNS analyzer), Inductively Coupled Plasma- Atomic Emission Spectroscopy and Atomic Absorption Spectroscopy. The nutritional uptake studies of lignin PSBs were done on *Vigna unguiculata* (L) Walp.

Results Musa acuminate produced handmade papers with the highest breaking length and burst factor (1160 m, 10.43 kg/cm2) followed by *Ficus citrifolia* (960 m, 7.2 kg/cm2), *Swietenia mahagoni* (480 m, 13.75 kg/cm2) and *Pinus roxburgii* (546 m, 4.0 kg/cm2) leaves. This lignin PSBs increased the growth of *Vigna unguiculata* (L) plant.

Conclusion High quality handmade papers were made from waste leaves. The lignin spent from pulping industry could be utilized as carriers to phosphate solubilizers.

Keywords Phosphate solubilizers, Lignin carriers, Hand-made paper, *Vigna unguiculata*

Introduction

The fertilizer industry experienced a permanent challenge for developing the efficiency of their products. An ideal fertilizer should have at least three characteristics, including facility for single application throughout the entire growing season; a high rate of yield return to the production input unit; and minimum lethal effects on soil, water, and atmospheric environments (Shoji and Kanno 1994; Trenkel 1997).

Pulp and paper industry was considered as one among the most polluted industry of the world (Thompson et al. 2001; Sumathi and Hung 2006). The wastewaters generated from production processes of this industry include high concentration of chemicals such as sodium hydroxide, sodium carbonate, sodium sulfide, bisulfites, elemental chlorine or chlorine dioxide, calcium oxide and hydrochloric acid (Sumathi and Hung 2006). Recent studies indicated that plant fiber cellulose possessed number of beneficial physical and chemical factors which had made it a very good raw material for textile and packaging industry (Vigneswaran et al. 2015). Lignocellulosic agricultural by-products had a copious and cheap source for cellulose fibers (Reddy and Yang 2005). The demand for wood fibre was recently solved by using increasing the amount of recycled fibres (Gokarneshan and Alagirusamy 2009). Many cottage

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✉ Gayathri Unnikrishnan
gay3avittam2012@gmail.com

[1] Department of Microbiology, Nehru Arts and Science College, Coimbatore, Tamil-Nadu, India
[2] Nehru Arts and Science College, Coimbatore, Tamil-Nadu, India

industries depend on manufactured paper bag based on recycled paper for their income. These bags could not meet the demand of user because of lower strength. Subsequently, pulping of waste leaf fiber was carried out by varying alkali charge, and cooking time at the boiling temperature.

Lignin was a waste material that could be ideal for application to soil and was one amongst the slow decomposed components of dead vegetation, and contributed a major fraction of the material that became manure as it underwent degradation. The resulted soil humus, in general, increased the photosynthetic productivity of plant. Moreover, this waste product, when embodied into soil, reduced the nitrification rate of urea (Wershaw 1982; Flaig 1984). In search for more accessible materials for bio-inoculants, lignin appeared as a feasible option. Lignin was an abundant and intrinsic component of the ligno-cellulosic biomass; it was innocuous, economic, and, most of all, a large renewable organic resource present in plants (Wang et al. 2018; Gillet et al. 2017; Xu et al. 2019). For many years, lignin had been recovered as a pulp and paper industry by-product (50 million tons annually) and, more recently, with the continued growth in global cellulosic ethanol production, larger quantities of residual lignin were being generated (1.0 kg/L of cellulosic ethanol). In 2017, an estimated 70 million tons of lignin were produced, given its low value; most of it was burnt for energy production (Supanchaiyamat et al. 2019). Consider the current increased accumulation of lignin, hence forth it became an ideal and suitable carrier for bio-inoculant production due to its organic nature and its inert matrix capabilities. There were several studies that supported preparation of lignin based slow/controlled released bio-fertilizers (Chen et al. 2020; Behin and Sadeghi 2016) for preventing ground water contamination particularly by nitrates during leaching process. In a recent study, *Azospirillum brasilense* was effectively attached to the lignin surface by biofilm formation, which led to a more reliable immobilization. This could be a promising solution for colonization and restoration of eroded soils that used lignin-immobilized bacteria (Tapia-Olivares et al. 2019).

Nevertheless, to our knowledge, neither any member of the genus *Meyerozyma* had been studied in detail for phosphate-solubilization and associated multiple plant growth promoting (PGP) abilities. *M. guilliermondii* had the ability to produce auxin which indicated this species also had importance in plant growth promotion and their prospective ability as agricultural crop growth enhancers (Jomar et al. 2017). The low availability of phosphorus in saline soil was attributed to its fixation with calcium, aluminium and iron which was then unavailable to plants (Sashidhar and Podile 2010). Hence, a study was conducted by Jiang et al. (2018) which first reported *Providencia rettgeri* sp as phosphate solubilizer.

This experimental research analysed the production process, structure, properties and suitability of waste leaf fibres for developing cost effective eco-friendly handmade paper products. Finally, the aim of this study was to investigate the suitability of lignin spent as carriers to phosphate solubilizers- *Meyerozyma gullerimondi* and *Providencia rettgeri*, which turned out to be a ground breaking work in lignin fertilizer production. Moreover, there were reports of only lignin used as hydrophobic coatings for slow release of nitrogenous fertilizers. Subsequently, in this study, the application of this lignin fertilizer on the growth parameters of crop *Vigna unguiculata* (L) was also recorded.

Materials and methods

Collection of samples

The waste dried leaves of plants like *Ficus citrifolia, Swietenia mahagoni, Musa acuminate, Pinus roxburgii* and *Acacia nilotica* found in Nehru Arts and Science College campus were chosen and collected for this study from a period between 2017 and 2019. Leaves were cleaned and cut into smaller pieces of 2 cm x 2 cm (length x breath). The technique used to make handmade papers were physical and chemical separation of cellulose and lignin (Hiebert 2006).

Handmade paper making

The workable parts (leaves) were separated by stripping. Selected parts were soaked in distilled water for overnight. Transferred wet stuff in a stainless-steel container and water was poured to cover it and boiled. This was followed by alkali application at constant pH of 9.0. The ratio used was 10 grams of caustic soda in 1 litre of neutral pH water equalled 1% solution, 15 grams of caustic in 1 litre of neutral pH water equalled 1.5% solution, 20 grams of caustic in 1 litre of neutral pH water equalled 2% solution. For soft fibre (*Ficus citrifolia* and *Musa acuminate*) 1% solution was used.

For medium strength fibre (*Swietenia mahagoni* and *Pinus roxburgii*) 1.5% solution was used then caustic solution was poured in stainless steel container and cooked the plant for 2-4 hours. The cooked fibre was washed in running water until it showed pH 7. The pulpy mass was cut into smaller pieces and beat either in hand grinder (kitchen mixer / grinder) or in Hollander beater.

The pulp was now poured in a tub or vat and water was added to give proper consistency for forming the sheet on the hand mold. The mold was placed inside a deckle. After the deckle had been taken from the mold, the mold was turned over and the sheet of paper was laid smoothly on a felt (woven woolen cloth). Here tub sizing was performed by passing through the dried paper into a solution of gelatin (or other size), contained in a bath or tub. The wet paper again was dried by solar drying (Chakraborty 2011).

Paper quality

Moisture content of the produced paper sheet was determined by placing one gram (1 g) of paper sheet on AD-4715 Infrared Moisture Determination Balance. The tensile strength and breaking length were tested for the paper sheet strips by using modified TAPPI (Technical Association of the Pulp and Paper Industry) 494 om-06 (Ilvessalo and Marja 1995) standard method for determining tensile properties of paper and paperboard. The GSM (grams per square meter) of each paper was measured.

Tensile strength and breaking length were calculated using formula given below.

Tensile strength (kN/m) = Maximum breaking force (kN)/width of paper strip (m).

Breaking length (km) = 102 000 × (Tensile strength (kN/m)/grammage (g/m^2).

Formulation of liquid bio-fertilizers and their shelf-life study

The lignin obtained after cellulose extraction of *Ficus citrifolia, Swietenia mahagoni, Musa acuminate, Pinus roxburgii, Azadirachta indica*, *Nerium oleander, Acacia nilotica, Bambusa vulgaris* and *Pinus roxburgii* was sterilized at 121°C at 15 lbs pressure. The sterilized medium was inoculated with liquid broth cultures (Potato Dextrose Broth) of phosphate solubilizers *Meyerozyma gullerimondi* and nutrient broth cultures of *Providencia retgiri.* The strains were revived from the soil of Composting unit of Kerala Agricultural University, Thrissur, Kerala. On 16SrRNA sequencing, they were identified as *Meyerozyma gullerimondi* and *Providencia retgiri.*

After inoculation, the fertilizers were fermented for seven days at 28℃. The fermented fertilizers were directly applied to Cowpea seedlings for 90 days. The phosphate solubilising liquid bio fertilizers were prepared in the ratio: for about 300 ml of lignin waste water, 50 ml each of potato dextrose agar (PDA) and nutrient broth (NB) contained phosphate solubilizers were added, 600 ml of sterilized nutrient broth was also added and made the fertilizer to 1 L and kept for fermentation for about 7 days. From this composition, 10 ml, 20 ml and 30 ml were dissolved in distilled water and applied as foliar and drenched to plants. At an interval of 15 days, shelf-life study was done which used serial dilution and plated on potato dextrose agar plates and nutrient agar plates (Cappuccino and Sherman 1992).

Field emission scanning electron microscopy (FESEM-EDX)

The surface morphology of lignin carrier extracted from *Ficus citrifolia, Swietenia mahagoni, Musa acuminate, Pinus roxburgii* and *Acacia nilotica* were observed by FE SEM (Quanta, FEG 250-Japan) and X-Ray spectroscopy. The samples were then adhered directly onto a cupreous stage and then sputter coated with a thin layer of gold. Digital photographs were captured with 10,000 times magnification at 10 kV and stored in TIFF format (Jiang et al. 2017; Helge et al. 2017). The bio-fertilizers produced after strain inoculation were tested for elemental mapping which used FESEM-EDX.

Physiochemical composition of residual effluent water

The lignin spent was tested and studied for its nutrient profile to determine their suitable nature for bio-fertilizer production as shown in Table 1.

Biometric observations of seedlings

Biometric observations were recorded at biweekly interval for three months. Plant height, number of leaves per plant, plant girth were measured. Vigour index was also calculated from the biometric observations.

Table 1 Methodology of residual effluent water analysis

Parameter	Method
pH	pH meter (FCO 1985)
Electrical Conductivity	EC meter (FCO 1985)
Total Nitrogen	Modified Kjeldhal digestion and distillation (Jackson 1958)
Organic Carbon	Ashing method (FCO 1985)
Total Phosphorous	Diacid digestion and estimation by Vanodomolybdate yellow colour method (Piper 1966)
Total Potassium	Diacid digestion and Flame photometric determination (Jackson 1958)
Total Calcium and Magnesium	Diacid digestion and determination using inductively coupled plasma atomic emission spectroscopy (ICP-AES)
Total Sulphur	Turbidimetric method (Bhargava and Raghupathy 1993)
Total Iron, Manganeese, Zinc and Copper	Diacid digestion and determination using Inductively coupled plasma atomic emission spectroscopy (ICP-AES)
Heavy Metals	Diacid digestion and ICP-AES

Statistical analysis

Data were subjected to analysis of variance (ANOVA) (Panse and Sukhatme 1985) which used statistical package 'MSTAT-C' package (Freed 2006). Wherever the F test was significant (at 5 % level), multiple comparison among the treatments was done with Duncan's Multiple Range test (DMRT).

Results and discussion

Characterization of handmade papers

Dry/Dead leaves were usually incinerated, which produced environmental pollution and toxicity to natural biome and ecosystem. In this study, we had designed an eco-friendly technique to process these leaves and create ecological, climate-friendly, green, environmentally sound, fuel-efficient, energy-efficient, non-polluting, organic handmade papers. In this experiment, we had also developed paper bags, baskets and dining table mats from *Ficus citrifolia* (Baniyan), *Swietenia mahagoni* (Mahogany), *Pennisetum purpureum* (CO_4 Grass) and *Musa acuminate* (Banana) leaves. Except banana leaves (Fagbemigun et al. 2016; Arafat et al. 2018; Ramdhonee and Jeetah 2017; Giri et al. 2018), all other samples were new to paper making industry. All of them had got legitimate score for breaking length and burst factor (Table 2). Comparing the properties of A_4 paper, which had breaking length of 2.56 km and burst index of 0.90 $kPam^2g^{-1}$ (Balbercak et al. 2017), the tested papers made from individual dry waste leaf samples could be used for writing and scribbling purpose as they had similar range of burst index and breaking length of A_4 sheet (1.1 km breaking length for *Musa acuminate* paper).

Caulfield and Guderson (1988) reported that the individual fibers, their arrangement, and the extent to which they were bonded to each other were key factors which contribute to tensile strength of handmade papers. Hence in our study, *Musa acuminate* produced handmade papers with highest breaking length and burst factor (1160 m, 10.43 kg/cm^2) followed by *Ficus citrifolia* (960 m, 7.2 kg/cm^2), *Swietenia mahagoni* (480 m, 13.75 kg/cm^2) and *Pennisetum purpureum* (546 m, 4.0 kg/cm^2). The breaking length of paper board handmade

Table 2 Properties of handmade paper made from leaf samples

Properties	Units	*Musa acuminate*	*Ficus citrifolia*	*Swietenia mahagoni*	*Pinus roxburgii*	*Acacia nilotica*
GSM	g/m^2	172.5	108.5	181.8	293	159.9
Moisture	%	4.0	4.0	4.0	6.0	6.0
Breaking length	meter	1160	960	480	546	300
Burst factor	Kg/cm^2	10.43	7.2	13.75	4.0	2.5

sheets is 2469 m (Xia et al. 2019; Patel 2016). On examining the above statements, our paper had only 960 m as highest breaking length, which made them unsuitable for wrapping purpose.

Designing of eco-friendly paper products

There were many types of paper bags, scribbling pads, paper baskets, paper files made from recyclable papers and similar materials. Among such materials were hand-made paper products made from waste leaf samples of *Ficus citrifolia, Swietenia mahagoni, Pinus roxburgii* and *Musa acuminate.* The paper sheets produced were non-perishable, moist resistant and non-collapsible. The products developed were baskets which were capable of standing on their base or bottom, which when filled, had a definite advantage over other types of baskets that had to be held in the hand when in use or otherwise must be supported by a holder. The durability of paper file and bags could be a best substitute to plastic covers in apparel industries. The main polluting constituent in normal pulp and paper mill wastewater were suspended solids, colour, foam, inorganics such as sodium carbonate, bicarbonate, chlorides and sulphates, toxic chemicals such as mercaptans and inorganic sulphides (Saadia and Ashfaq 1970). Once these eco-friendly handmade paper products were produced, there was zero discharge wherever feasible. The products made from extracted cellulose of dead-dry waste leaves were shown in Fig. 1.

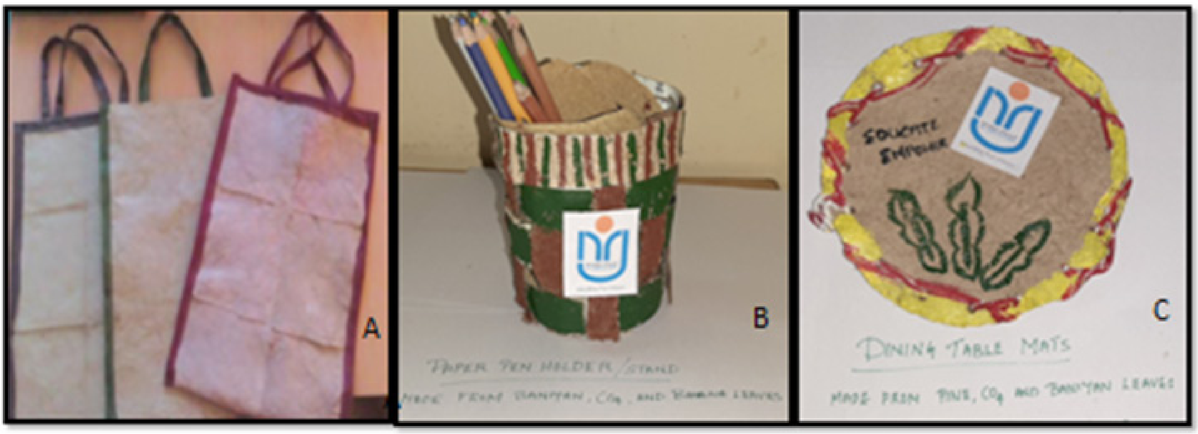

Fig. 1 Eco-friendly paper products made from (A) Paper bag from *Ficus citrifolia, Swietenia mahagoni, Musa acuminate* leaves; (B) Paper pen holder made from *Musa acuminate, Pinus roxburgii* and *Acacia nilotica* leaves; (C) Dining table mats made from *Swietenia mahagoni* and *Musa acuminate* leaves

Qualitative estimation of essential plant nutrients in lignin carrier biofertilizer

Field emission scanning electron microscope (FE -SEM), elemental mapping, and energy dispersive X-ray spectroscopy (EDX) of lignin carriers were analyzed with an electron microscope, FE SEM (Quanta, FEG 250-Japan) instrument, operated at 10 kV. The selected plants, *Ficus citrifolia, Swietenia mahagoni, Musa acuminate, Pinus roxburgii* and *Acacia nilotica* grew indigenously in fair quantities and some of them had been reported traditionally for the preparation of handmade papers. The conversion rate of all these lignin extracts to bio-fertilizers by *Providencia rettgeri* and *Meyerozyma gullerimondi* was confirmed through FESEM images in Fig. 2. This Fig clearly depicted how the lignin extract was completely decomposed by phosphate solubilizers after 6 months. More recently, there was a study that had screened the crude extracts derived from these plants for various in vitro biological activities and their standardizations (Santhosh et al. 2019). Neither these plants nor their extracts have, however, been tested for their elemental composition. Here, we present, for the first time, the results of elemental composition of crude lignin extracts derived from these plants after cellulose extraction and their respective bio-fertilizers produced using FESEM-EDX technique. The spectra obtained for these extracts were given in Fig. 3 while their elemental compositions were listed in Table 3.

Table 3 Elemental analysis (EDAX) of crude extracts and its bio-fertilizers

Lignin Extracts	**Elemental Composition (%)**																						
	C		**O**		**Na**		**N**		**Cl**		**P**		**S**		**K**		**Zn**		**Ca**		**Mg**		
	W%	**A%**	**W%**	**A%**	**W%**	**A%**	**W%**	**A%**	**W%**	**A%**	**W%**	**A%**	**W%**	**A%**	**W%**	**A%**	**W%**	**A%**	**W%**	**A%**	**W%**	**A%**	
Swietenia mahagoni	C	13.35	21.96	35.74	44.14	11.87	10.20	1.45	2.05	3.59	2.00	2.94	0.89	1.06	0.65	10.51	5.31	2.94	0.89	3.18	1.57	1.90	1.54
	B	28.98	43.17	24.63	27.54	10.72	8.34	4.62	5.90	9.10	4.59	0.32	0.18	0.99	0.55	5.77	2.64	3.36	0.92	3.20	1.43	1.01	0.74
Pinus roxburgii	C	52.41	61.84	29.36	26.01	5.91	3.64	5.61	5.68	3.08	1.23	1.74	0.80	1.63	0.72	0.11	0.04	0.07	0.02	0.07	0.02	0.01	0.01
	B	49.74	61.43	20.49	19.00	8.79	3.68	8.42	8.91	6.78	4.37	2.47	1.19	2.02	0.93	1.28	0.49	0.01	0.00	0.00	0.00	0.00	0.00
Acacia nilotica	C	49.06	58.91	31.34	28.25	8.74	5.48	4.62	4.76	3.96	1.61	1.00	0.46	0.71	0.32	0.33	0.12	0.12	0.03	0.07	0.04	0.06	0.02
	B	60.20	68.40	27.40	23.37	4.35	4.23	3.62	2.15	1.92	0.84	1.17	0.45	1.00	0.43	0.30	0.11	0.02	0.01	0.02	0.01	0.00	0.00
Ficus citrifolia	C	66.81	72.84	33.19	27.16	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	Nd
	B	56.13	63.79	39.13	33.39	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	Nd

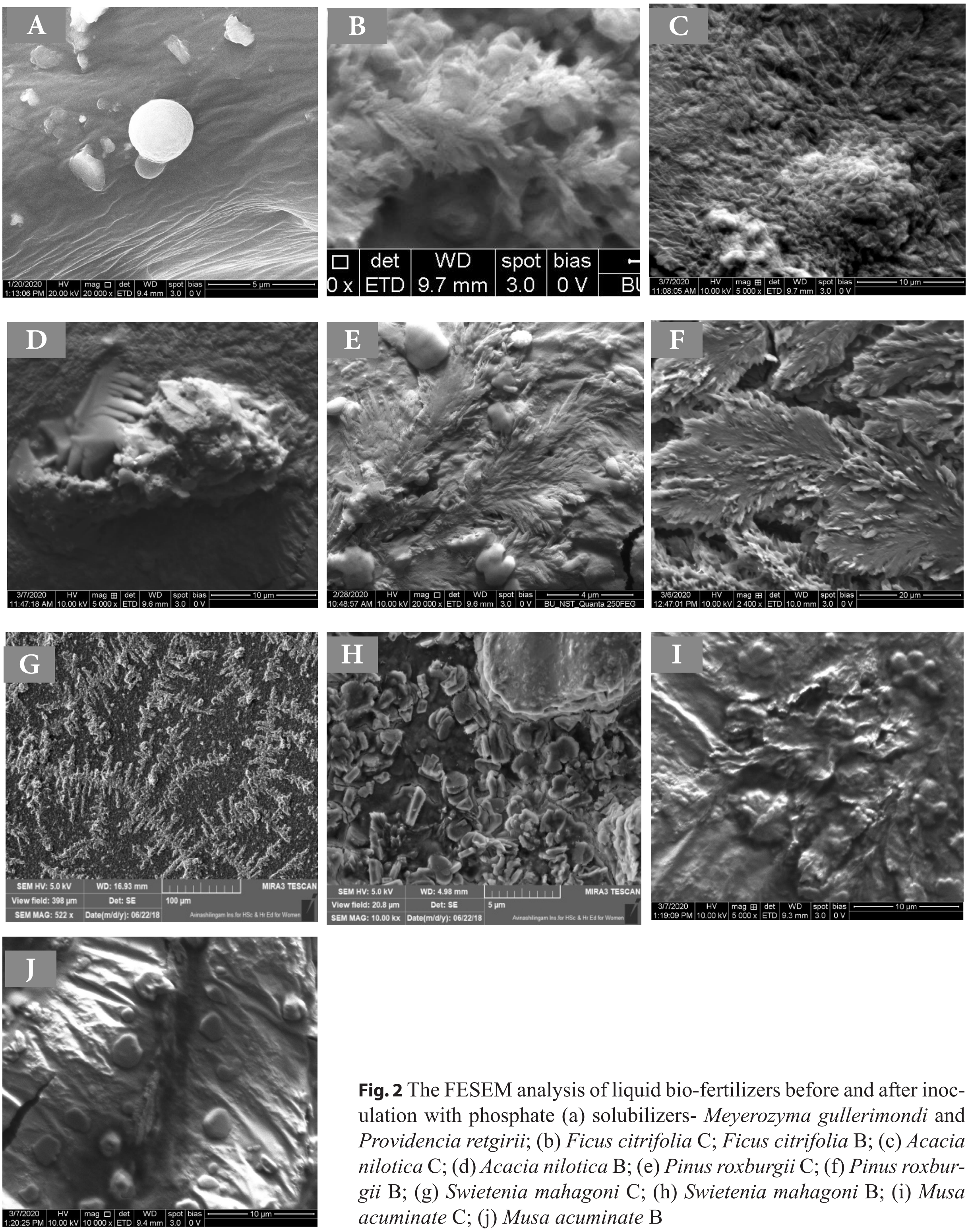

Fig. 2 The FESEM analysis of liquid bio-fertilizers before and after inoculation with phosphate (a) solubilizers- *Meyerozyma gullerimondi* and *Providencia retgirii*; (b) *Ficus citrifolia* C; *Ficus citrifolia* B; (c) *Acacia nilotica* C; (d) *Acacia nilotica* B; (e) *Pinus roxburgii* C; (f) *Pinus roxburgii* B; (g) *Swietenia mahagoni* C; (h) *Swietenia mahagoni* B; (i) *Musa acuminate* C; (j) *Musa acuminate* B

As can be seen from Fig. 3(g), the crude extract derived from *Pinus roxburgii* showed the presence of various elements such as C, O, Na, N, Cl, P, S, K, Zn, Ca, and Mg in which S was in the highest percentage (> 80 %) than other extracts followed by P which was also detected in higher amounts comparatively (Table 3). Highest amount of N was detected in *Musa acuminate* bio-fertilizer, Fig. 3(d). The maximum concentration of Cl, K, Zn, Ca and Mg were detected in *Swietenia mahagoni* bio-fertilizer, Fig. 3(I). The O, Na and N content of hair waste water after extraction was high in comparison with other bio-fertilizers produced. The crude extract and the bio-fertilizers

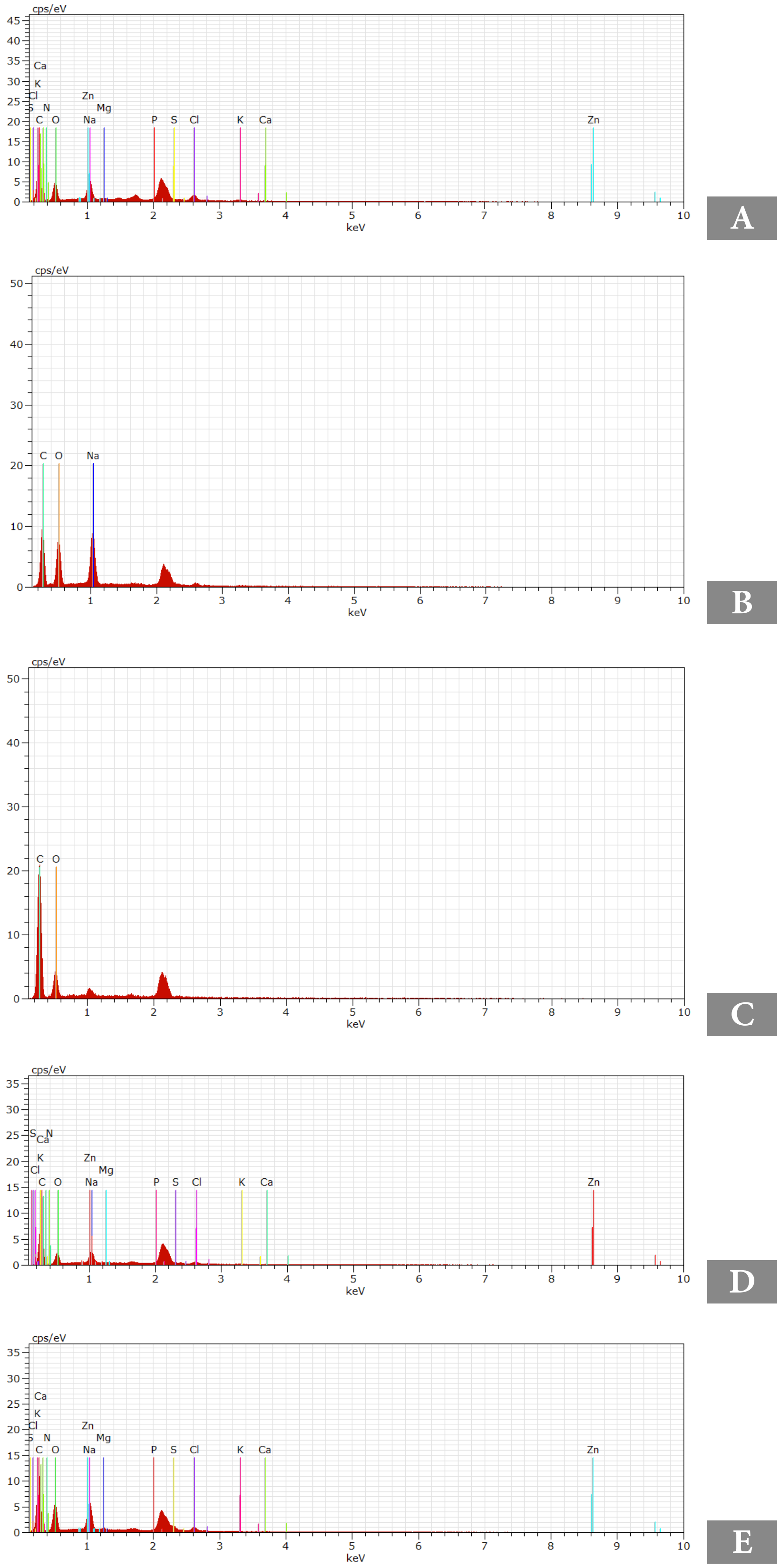

Fig. 3 FESEM-EDX spectra for elemental analysis of the crude extracts and its bio-fertilizers Derived from the selected plants of college campus are as follows (A) *F. citrifolia* C; (B) *F. citrifolia* BF; (C) *Musa acuminate* C; (D) *Musa acuminate* BF; (E) *Acacia nilotica* C; (F) *Acacia nilotica* BF; (G) *Pinus roxburgii* C; (H) *Pinus roxburgii* BF; (I) *Swietenia mahagoni* BF; (J)*Swietenia mahagoni* C

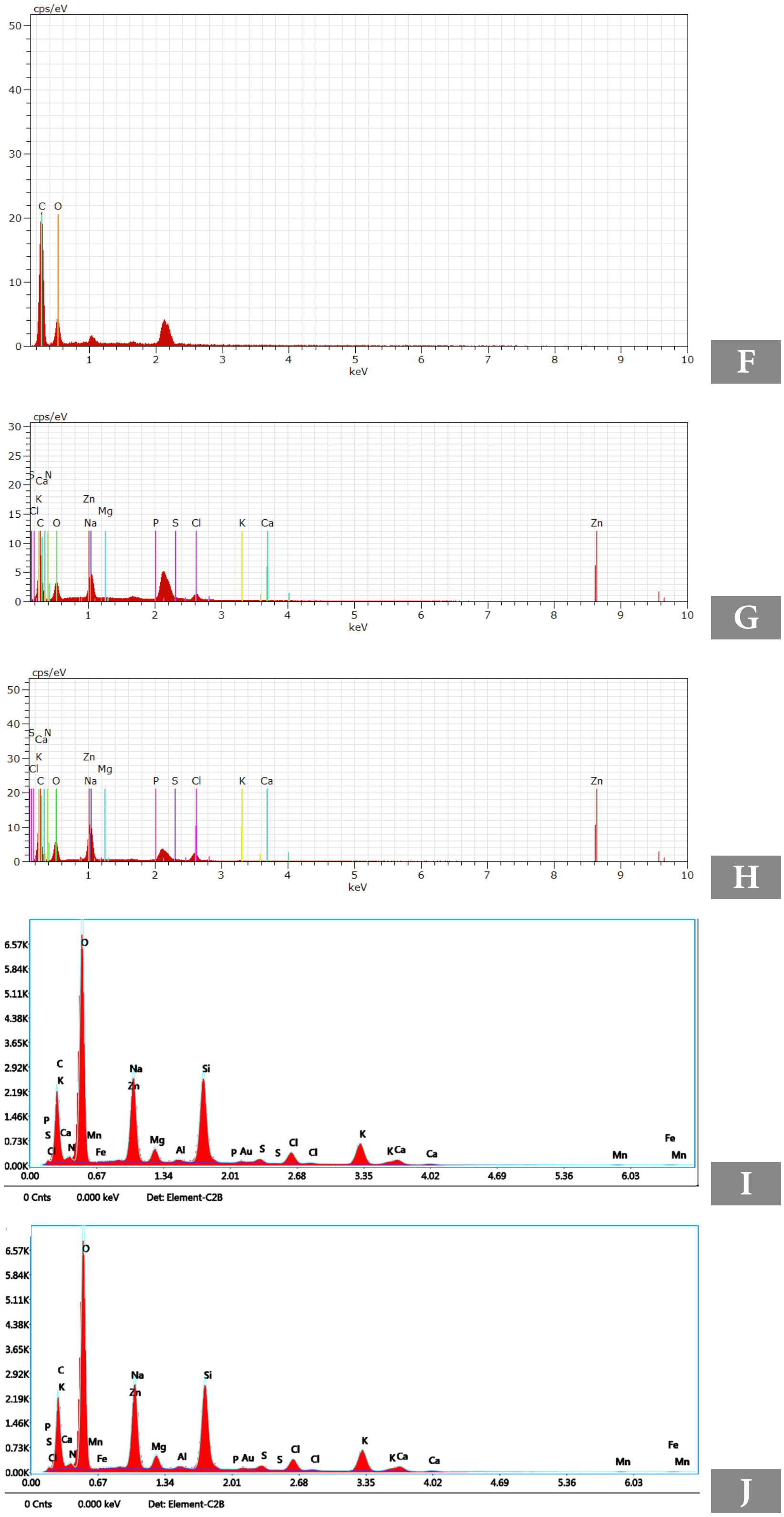

Fig. 3 Continues

made from *Ficus citrifolia* and *Acacia nilotica* were found to have lower nutrient index compared to *Swietenia mahagoni, Musa acuminate* and *Pinus roxburgii.* The percentage compositions of all these elements were shown in Table 3. Henceforth, the qualitative compositions of all carriers were profiled using FESEM EDX. They were found to be a sustainable medium for growth and development of microbial fertilizers. The corresponding macronutrient and micronutrient profiling also suggest its growth promoting boosters for plant growth and development. This FESEM-EDX study also describes the particle morphology of lignin carrier before bio-fertilizer production and after inoculated phosphate solubilizers, lignin carriers were identified as bio-fertilizers.

On performing FE-SEM EDX, accurate levels of essential nutrients for plant growth were also estimated qualitatively (Lakshmanan et al. 2018; Saha et al. 2020). The suitability of this lignin wastewater as a carrier to phosphate solubilizers were also tested quantitatively which determined their nutritional composition by AAS and ICP-AES (Balaram 2020; Chojnacka and Mikulewicz 2019). Thereby, these parameters were experimented, which proved the ability of this wastewater as carriers to bio-fertilizers. Moreover, the proposed system was also able to recover 90%, 75%, 20% and 40% of total C, N, P and K content in the initial waste, respectively. Compared to the previous report of Mia et al. (2018) who quantitatively determined nutrients from municipal organic waste and could recover only 46%, 54%, 54% and 61% of total C, N, P and K content in

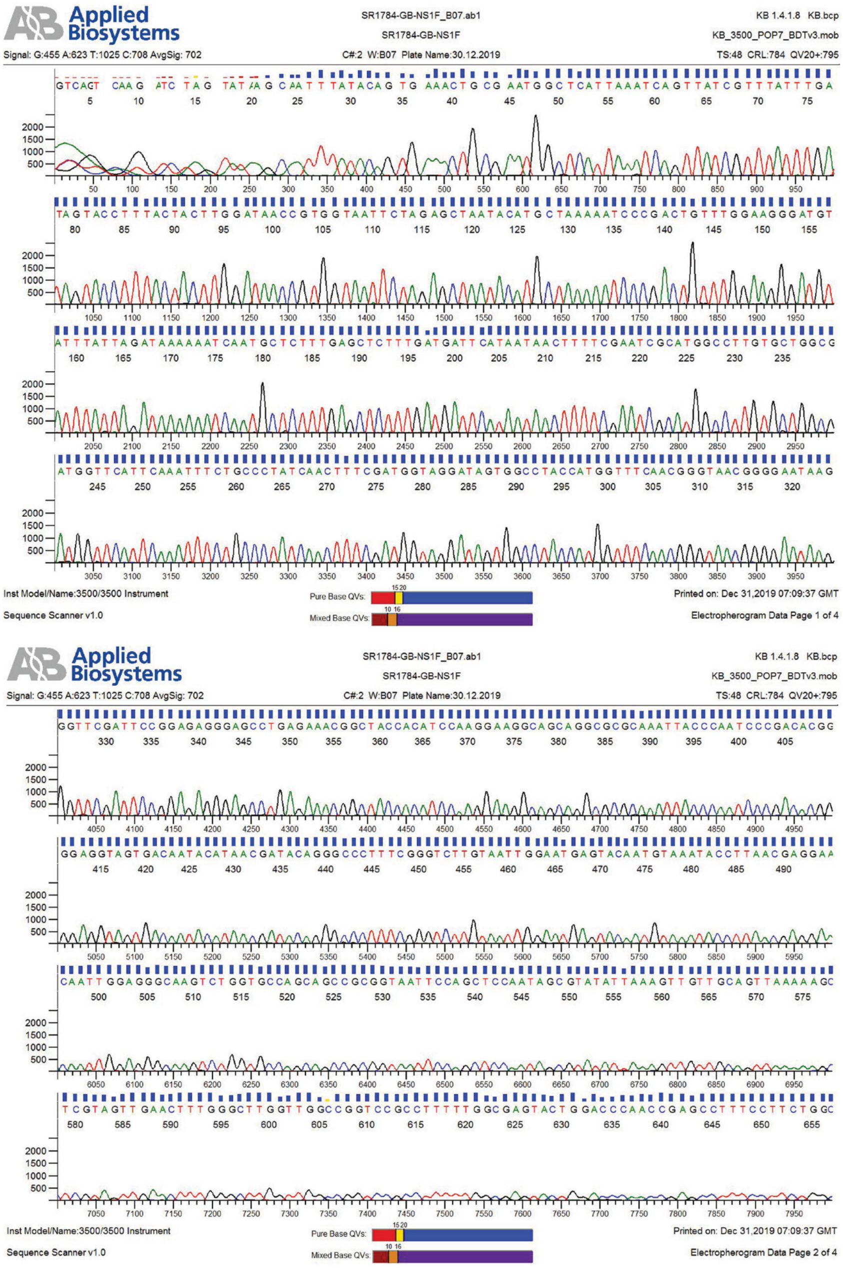

Fig. 4 Electropherogram of *Meyerozyma guilliermondii*

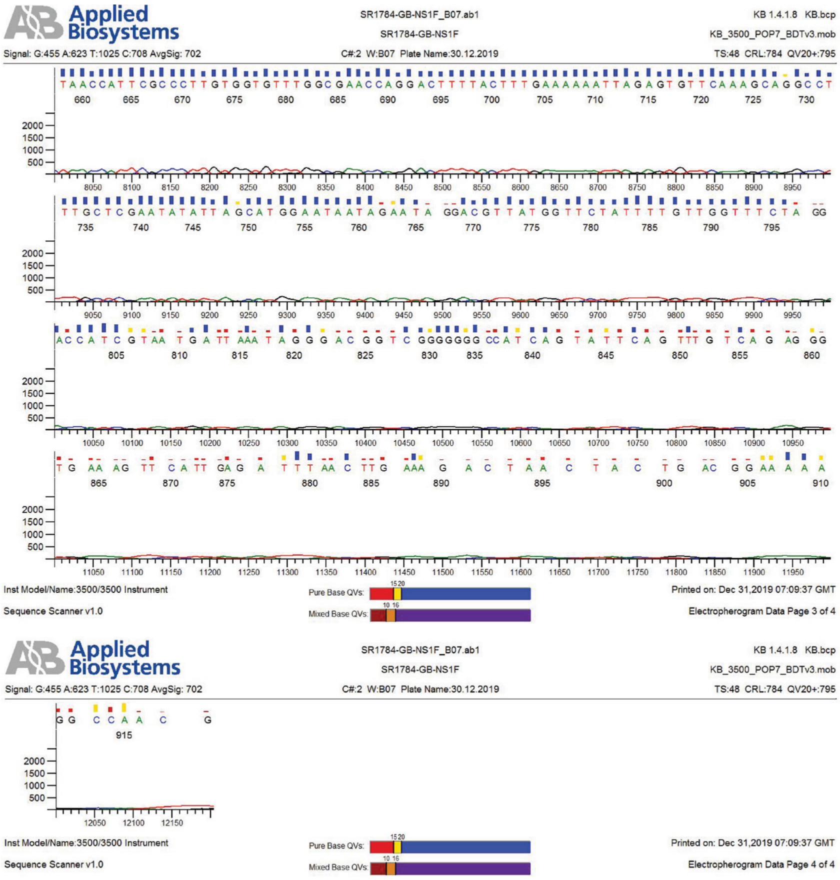

Fig. 4 Continues

the initial waste, in our experiment, the waste recovery percentage was high.

More recently, we screened the crude extracts derived from these plants for various in vitro biological activities and their standardizations (Santhosh et al. 2019). Neither these plants nor their extracts have, however, ever been tested for their elemental composition. Here, we present, for the first time, the results of elemental composition of crude extracts from these plants after cellulose extraction and their respective bio-fertilizers produced were tested which used FE-SEM-EDX technique.

Formulation of liquid bio-fertilizers and their shelf-life study

According to FAO-fertilizer and plant nutrition bulletin, for an average nutrient application of about 92 kg/ha, it should contain 65 percent N, 25 percent P_2O_5 and 10 percent K_2O (Roy et al. 2006). Considering the above scientific ratio, the FESEM-EDX data showed the phosphorous content of all the plant extract fertilizers and control spent which was below essential limits (>3 percent). Consequently, phosphorous solubilizing microbes were isolated and revived from the composting unit soil (Kerala Agricultural University, Composting unit) and sequenced. After sequencing, the organisms were identified as *Providencia rettgeri* and *Meyerozyma guilliermondii* with 98 percent similarity indexes. Electropherograms of *Meyerozyma guilliermondii* was shown in Fig. 4 and *Providencia rettgeri* was depicted in Fig. 5. So, the quantitative estimation of their essential nutrients was done using Atomic Absorption Spectroscopy (AAS), Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) and Carbon Hydrogen Nitrogen Sulphur Analyzer (CHNS). According to the data in Table 4, the bio-fertilizer *Acacia nilotica* was rich in Carbon, Iron, Zinc and Phosphorous. The bio-fertilizer *Sweitenia mahogany* was rich in Hydrogen and Potassium, *Pinus roxburghii* was rich in Chlorine, Copper and Manganese. Heavy metals such as Chromium, Nickel and Lead were below detectable levels in all fertilizers according to Proposed Fertilizer Act, 2003 (IFDC 2003).

The shelf-life studies of carrier-based bio-fertilizers were done from August 2019 to February 2020. From Table 4, the initial population of *Providencia rettgeri* was 9.7 x10^7 in Nutrient Agar and *Meyerozyma* *gullerimondi* was 9.2x10^4 in Potato Dextrose Agar. The viability of microorganisms was evaluated on monthly intervals up to February 2020. The microbial analysis revealed that there was a decline in the population of *Providencia rettgeri* from December (5.3 x10^7CFU ml^{-1}) to February (1.5 x10^7 CFU ml^{-1}). Similar count was also generated for *Meyerozyma gullerimondi*. Level of contaminants observed were 3.2 x10^7, 1.5 x10^7 CFU ml^{-1} during last two months, i.e., January and February. The quality of all four carriers was good till fourth month, after which the bio-fertilizers started to attain contamination. The carrier with maximum sustainable shelf life for bio-fertilizer, *Providencia rettgeri* and *Meyerozyma gullerimondi* was *Acacia nilotica.* Similar investigations were done by Bhavya et al. (2017).

In our experiment, the phosphate content of lignin waste water was comparatively low; hence, we had selected a newly reported phosphate solubilizer *Providencia rettgeri* (Jiang et al. 2018) which had a phosphate-solubilizing index (PSI) between 1.1 and 2.58.

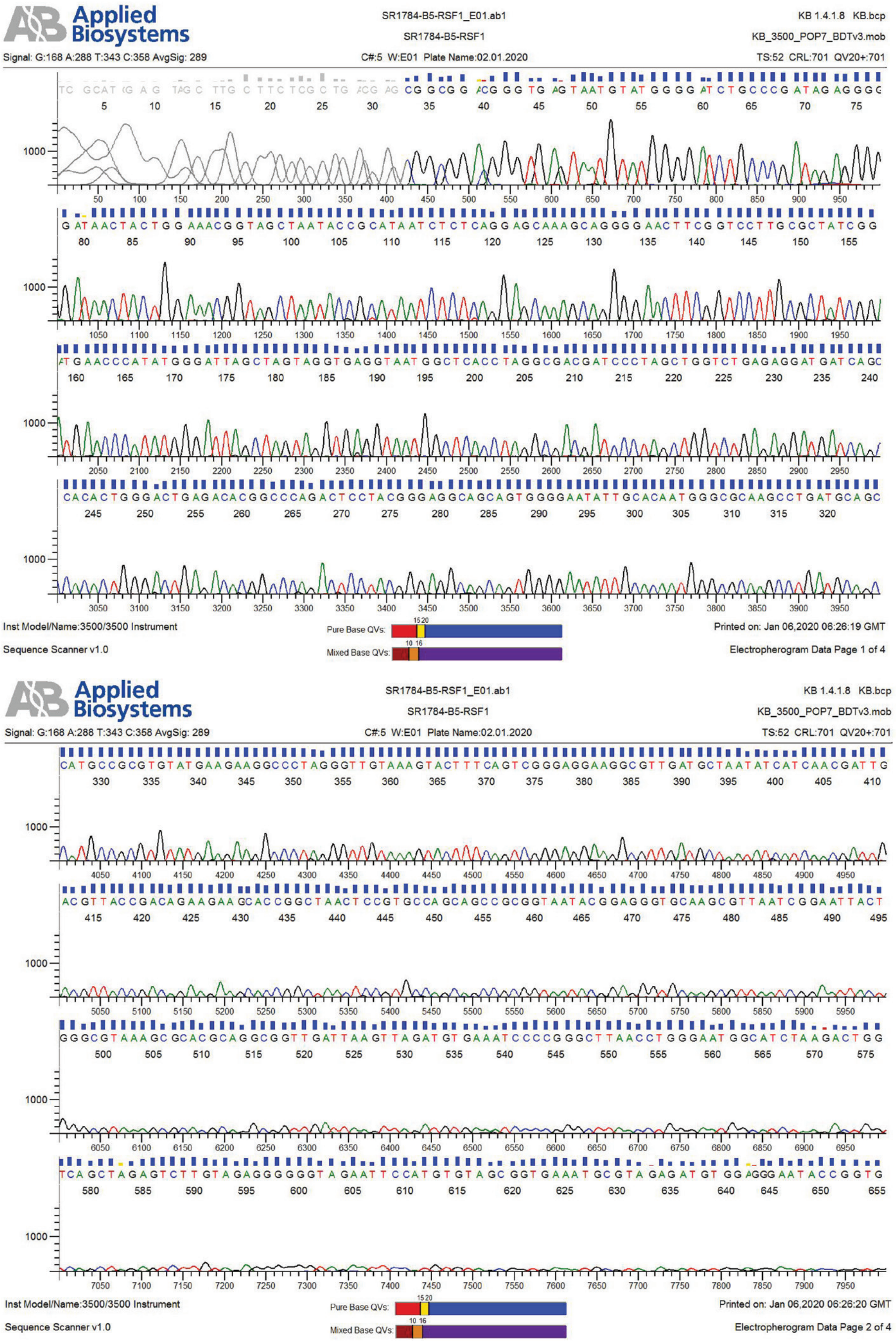

Fig. 5 Electropherogram of *Providencia rettegiri*

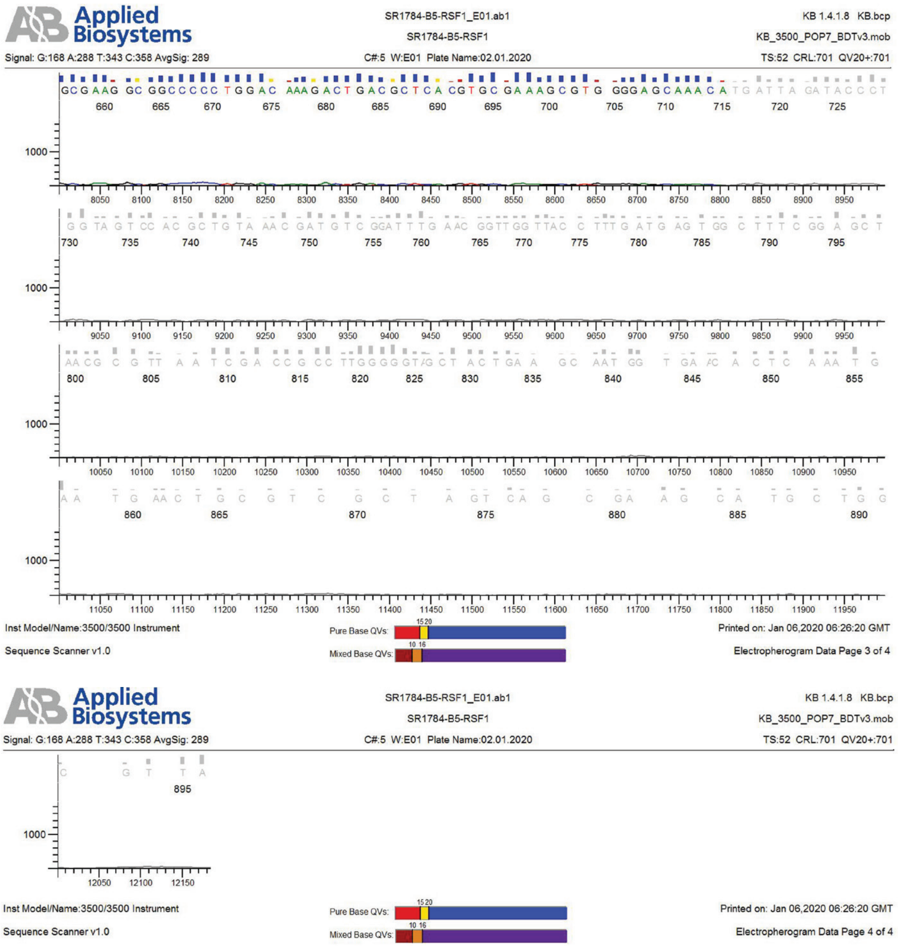

Fig. 5 Continues

The second strain chosen was *Meyerozyma guilliermondii* an under-exploited phophate solubilizing soil yeast (Nakayan et al. 2013). These organisms were isolated from leaf composting unit of Kerala Agricultural University, Vellanikara, Thrissur. Hayati et al. (2019) reported that the organic leaf compost when combined with phosphate solubilizing bacteria increased the growth and yield of plants. Considering this possibility, in our study, *Ficus citrifolia* (Baniyan)*, Swietenia mahagoni* (Mahogany*), Pinus roxburgii* (Pine*)* and *Acacia nilotica* (Acacia) lignin extracts were used for testing their carrier efficiency for phosphate solubilizing bio-fertilizers (PSBs) applied on cowpea (*Vigna unguiculata)* for first time. Comparing a recent report that used agricultural residues as carrier to bio-fertilizer production keeping *Azospirillum brasilense* as model organism (Tapia-Olivares et al*.* 2019; Sarin and Riddech 2018). Hence forth, in this research, phosphate solubilizers: *Providencia rettgeri* and *Meyerozyma guilliermondii* were used as model organism for bio-inoculant production which had maximum fertigation efficiency when applied at a dosage of 7x10^7 CFU per 30 ml of lignin carrier.

Biometric observations

The bio-fertilizers made from different lignin carrier used were evaluated for the maximum manorial value. It was found that *Acacia nilotica* bio-fertilizer contained the maximum nutritional value for all essential elements. Conclusively for about 10 ml of paper lignin carrier bio-fertilizer, corresponding nutrient compositions were given in Table 5. In order to study the effect of this lignin carrier fertilizer on germination of *Vigna unguiculata,* different treatments at various dosages of biofertilizers made were studied. Following this, *Acacia nilotica* and *Swietenia mahagoni* biofertilizers were studied separately on Cowpea plants whereas *Pinus roxburgii* and *Ficus citrifolia* were mixed together and applied to Cow pea plants as they were deficient in individual nutrients compared to *Acacia nilotica* and *Swietenia mahagoni* carriers.

The data presented in Fig. 6 indicated germination percent of *Vigna unguiculata* in the range of 49.90 % to 60.46 % for variety of *Acacia nilotica,* 49.56% to 60 .08% for *Swietenia mahagoni* and 39.20% to 60.08

Table 4 Shelf life of carrier-based phosphate solubilizing bio-fertilizers produced from lignin carrier

Samples (Carriers)	**Phosphate Solubilizers**	**First Month TPC (CFU ml^{-1})**	**Second Month TPC (CFU ml^{-1})**	**Third Month TPC (CFU ml^{-1})**	**Fourth Month TPC (CFU ml^{-1})**	**Fifth Month TPC (CFU ml^{-1})**	**Sixth Month TPC (CFU ml^{-1})**
Ficus citrifolia	*Providencia rettgiri* (NA)	5.4x10^{7}	5.4 x10^{7}	5.0 x10^{7}	3.4x10^{7}	1.6x10^{7} TFTC	0.9 x10^{7} TFTC
	Meyerozyma guilliermondii (PDA)	5.0x10^{4}	5.0 x10^{4}	4.8x10^{4}	3.8x10^{4}	1.9x10^{4} TFTC	0.3 x10^{4} TFTC
	Combined	12x10^{5}	12x10^{5}	9.0x10^{5}	8x10^{5}	TFTC	TFTC
Acacia nilotica	*Providencia rettgiri* (NA)	9.7 x10^{7} TNTC	9.5x10^{7}	8.8x10^{7}	5.3 x10^{7}	3.2 x10^{7}	1.5 x10^{7} TFTC
	Meyerozyma guilliermondii (PDA)	9.2x10^{4} TNTC	9.0x10^{4}	8.5 x10^{4}	6.2 x10^{4}	4.0 x10^{4}	2.1 x10^{4} TFTC
	Combined	20 x 10^{5}	20 x 10^{5}	16 x 10^{5}	13 x 10^{5}	TFTC	TFTC
Swietenia mahagoni	*Providencia rettgiri* (NA)	8.9 x10^{7} TNTC	8.6 x10^{7}	8.6 x10^{7}	5.3 x10^{7}	3.0 x10^{7}	1.9 x10^{7} TFTC
	Meyerozyma guilliermondii (PDA)	8.3 x10^{4} TNTC	8.0 x10^{5}	7.5 x10^{5}	5.0 x10^{5}	2.3 x10^{4} TFTC	0.6 x10^{4} TFTC
	Combined	20 x 10^{5}	18 x 10^{5}	15 x 10^{5}	11 x 10^{5}	TFTC	TFTC
Pinus roxburgii	*Providencia rettgiri* (NA)	6.0 x10^{7}	6.0 x10^{7}	5.8 x10^{7}	4.1 x10^{7}	2.7 x10^{7} TFTC	0.6x10^{7} TFTC
	Meyerozyma guilliermondii (PDA)	5.7 x10^{4}	5.7 x10^{4}	5.4 x10^{4}	3.6 x10^{4}	1.9 x10^{4} TFTC	0.4 x10^{4} TFTC
	Combined	11 x 10^{5}	11 x 10^{5}	8 x 10^{5}	8 x 10^{5}	TFTC	TFTC

NA: Nutrient agar, PDA: Potato Dextrose Agar, TNTC: Too Numerous to count, TFTC: Too Few to Count,TPC: Total plate count, CFU: Colony forming unit

% for *Pinus roxburgii* and *Ficus citrifolia*. The treatment with *Acacia nilotica* registered maximum values among the three varieties. This may be due to better mobilization of nutrients, hydrolyzation of reserved carbohydrates and better enzyme activity. The layout of the experiment had 5 treatments applied to *Vigna unguiculata* as $T_{1:}$ Storage in shade (Control), $T_{2:}$ Foliar and drenching of 10 ml paper effluent bio-fertilizer in 1liter of water per plant, $T_{3:}$ Foliar and drenching of 20 ml paper effluent bio-fertilizer in 1liter of water per plant, $T_{4:}$ Foliar and drenching of 30 ml paper effluent bio-fertilizer in 1 litre of water per plant, $T_{5:}$ Foliar and drenching of 10 ml effective microorganism (EM) solution in 1 litre of water per plant.

Seedling vigour was also significantly influenced by the treatments of three varieties. As the seedling height for *Acacia nilotica* was comparatively higher than that of *Swietenia mahagoni* and *Ficus citrifolia* (Fig. 7), the vigour index registered higher value for *Acacia nilotica.* This could be due to the presence of beneficial microbial biomass *Providencia rettgeri* and *Meyerozyma gullerimondi.* Hence forth, *Acacia nilotica* was enriched with maximum nutrients to act as carriers for PSBs used.

The effect of treatments on seedling height of *Vigna unguiculata* was evident in this variety of Cowpea. The highest seedling height (64.30) at 90 days was recorded in the T_5 treatment with 10 ml EM solution in 1 litre of water. Comparing to the control plant in shade (50.0 cm in 90 days), the treatments with paper effluent bio-fertilizer had shown maximum increase (60.40 cm in 90 days) with 30 ml of paper effluent bio-fertilizer in1 litre of water. This gradual increase in seedling length confirmed the effectiveness of paper effluent fertilizer which when applied in large quantity showed increased plant height. EM promoted germination, growth, flowering, fruiting and ripening of crops. This market fertilizer contained mixture of *Lactobacillus casei, Rhodopseudomonas palustris,* and *Saccharomyces cerevisiae* with pulses and jaggery. On comparing the activity of paper effluent fertilizer with EM solution, similar activity was observed significantly in terms of seedling height of *Vigna unguiculata* [CD (0.05) = 0.493]. Among the three-paper effluent fertilizer, *Swietenia mahagoni* (Fig. 8b) recorded maximum seedling height followed by *Acacia nilotica* (Fig. 8a)*, Ficus citrifolia* and *Pinus roxburgii* mixture (Fig 8c).

Seedlings of cowpea was mainly used in tissue culture to develop bushy varieties with maximum yield, for this purpose sufficient girth was an important prerequisite. Assessment of different treatments on seedling girth revealed that the treatments were significantly different in initial two months (Fig. 10) but at 90 days after planting, the highest girth was re-

Table 5 Major nutrients and heavy metal composition of different bio-fertilizers

Samples	C%	H%	N%	S%	Cl (ppm)	Cu (ppm)	Fe (ppm)	Mg (ppm)	Mn (ppm)	Zn (ppm)	PO_4 (ppm)	K (ppm)	Cr (ppm)	Ni (ppm)	Pb (ppm)
Pinus roxburgii	0.56	18.35	0.12	ND	14.81	0.151	2.389	14.75	0.088	0.020	7.75	0.50	188	BDL	BDL
Ficus citrifolia	0.31	18.68	0.23	ND	7.574	0.214	2.683	2.523	0.018	0.030	3.40	ND	1.36	BDL	BDL
Acacia nilotica	1.07	17.96	0.12	ND	22.05	0.208	12.03	29.18	0.470	0.246	24.13	0.12	BDL	BDL	BDL
Swietenia mahagoni	0.29	20.00	0.14	ND	3.406	0.092	2.465	2.162	0.024	0.177	1.664	5.77	1.1	BDL	BDL
Pinus roxburghii	0.21	19.55	0.12	ND	22.11	1.159	3.469	13.82	0.027	0.084	1.203	ND	1.1	BDL	BDL

BDL: Below Detectable Levels, ND: Not Detected

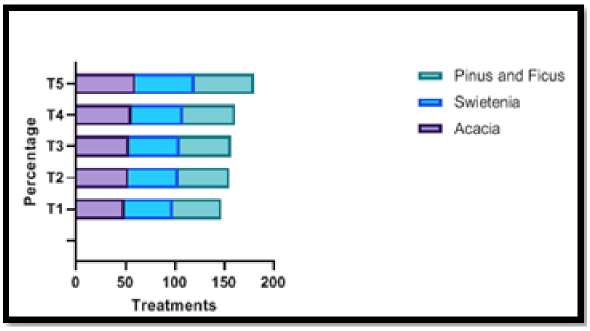

Fig. 6 Graph showing germination % of cowpea on application of phosphate solubilizer in lignin extract carrier

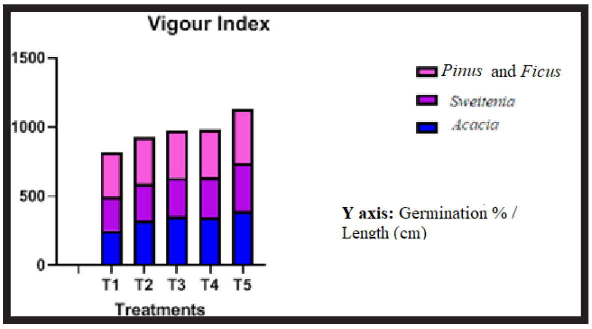

Fig. 7 Graph showing vigour index of cowpea on application of phosphate solubilizer in lignin extract carrier

corded in seedlings of treatment with 10 ml EM solution followed by 30 ml of paper effluent bio-fertilizer treatment.

The effect of treatment on number of leaves of seedling, variety of *Vigna unguiculata* was significant at 60 days of planting and found to be non-significant thereafter. The highest number of leaves was recorded in T_5 (10 ml EM solution) at 60 days of *Swietenia mahagoni* application (Fig. 9b). This w\as followed by *Acacia nilotica* bio-fertilizer (Fig. 9a) and *Ficus citrifolia* and *Pinus roxburgii* mixture (Fig. 9c).

The girth of cow pea was maximum for *Acacia nilotica* bio-fertilizer (Fig. 10a), *Ficus citrifolia* and *Pinus roxburgii* mixture (Fig. 10c) and *Swietenia mahagoni* (Fig. 10b). Therefore, bio-fertilizer and chemical fertilizer application had synergetic effect on P content. Increasing the availability of P in soils with inoculation of PGPR, which may lead to increased P uptake and plant growth, was reported by many researchers. Çakmakçi et al. (2007; 2009) reported that phosphate solubilizing, N2-fixing PGPR increased the uptake of P in spinach and wheat plants.

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Mehrvarz et al. (2008) reported that phosphate solubilising bacteria contained biological fertilizers which had the ability to supply the required phosphorus to stimulate the growth of the plant. Subsequent study was done by Zarabi et al. (2011) which showed phosphate solubilising microorganisms increased the growth and phosphorous absorption in maize plant. Similarly, Shabani et al. (2011) stated that synergetic growth effect due to plant phosphorous concentration was observed by integrated application of P solubilizing bacteria, *Mycorrhiza* fungi, and N-fixing bacteria. This result could be explained by PGPR positive contribution in the efficiency of nutrient absorption by plants, which improved uptake of minerals such as phosphorous.

The lignin extract obtained after handmade paper making of *Ficus citrifolia* (Baniyan)*, Swietenia mahagoni* (Mahogany), *Pinus roxburgi* (Pine) and *Acacia nilotica* (Acacia) were tested for fertilizing efficiency on *Vigna unguiculata* (Cowpea). The growth parameters tested were germination percent, vigour index, seedling length, number of leaves and seedling girth. The higher the concentration of lignin extract fertilizer in water (3:100), the highest growth was observed. An organic fertilizer, like EM solution was also comparatively tested with lignin extract fertilizer. In a recent study, the use of the bio-fertilizer had shown to be advantageous for

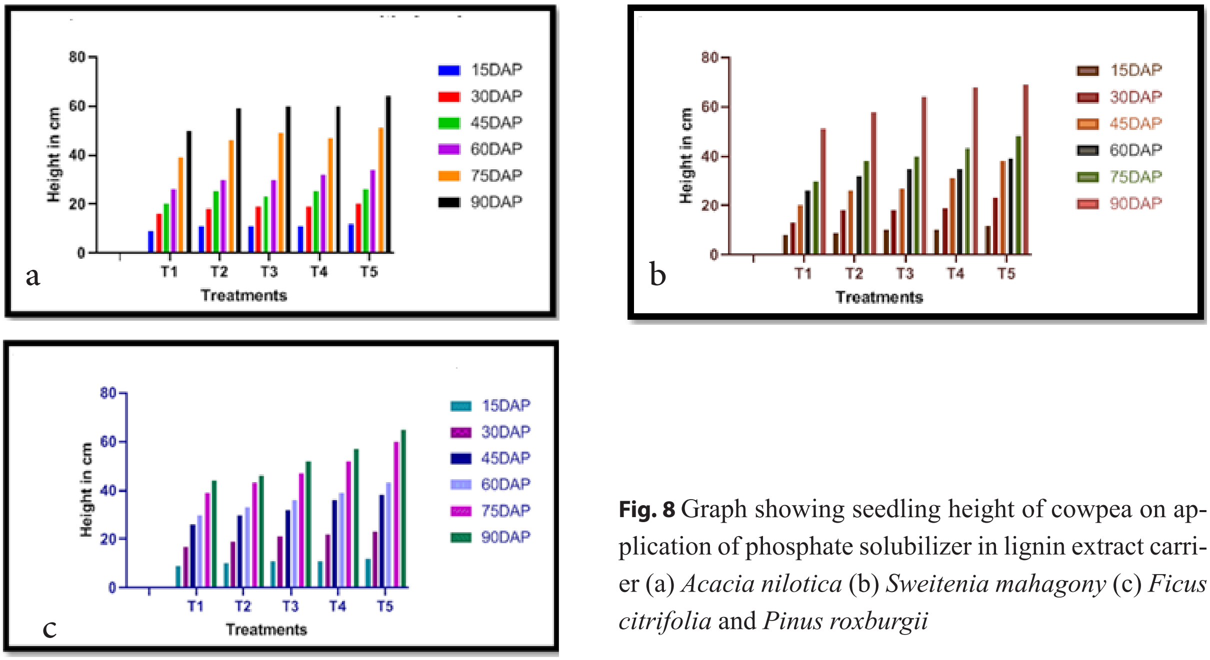

Fig. 8 Graph showing seedling height of cowpea on application of phosphate solubilizer in lignin extract carrier (a) *Acacia nilotica* (b) *Sweitenia mahagony* (c) *Ficus citrifolia* and *Pinus roxburgii*

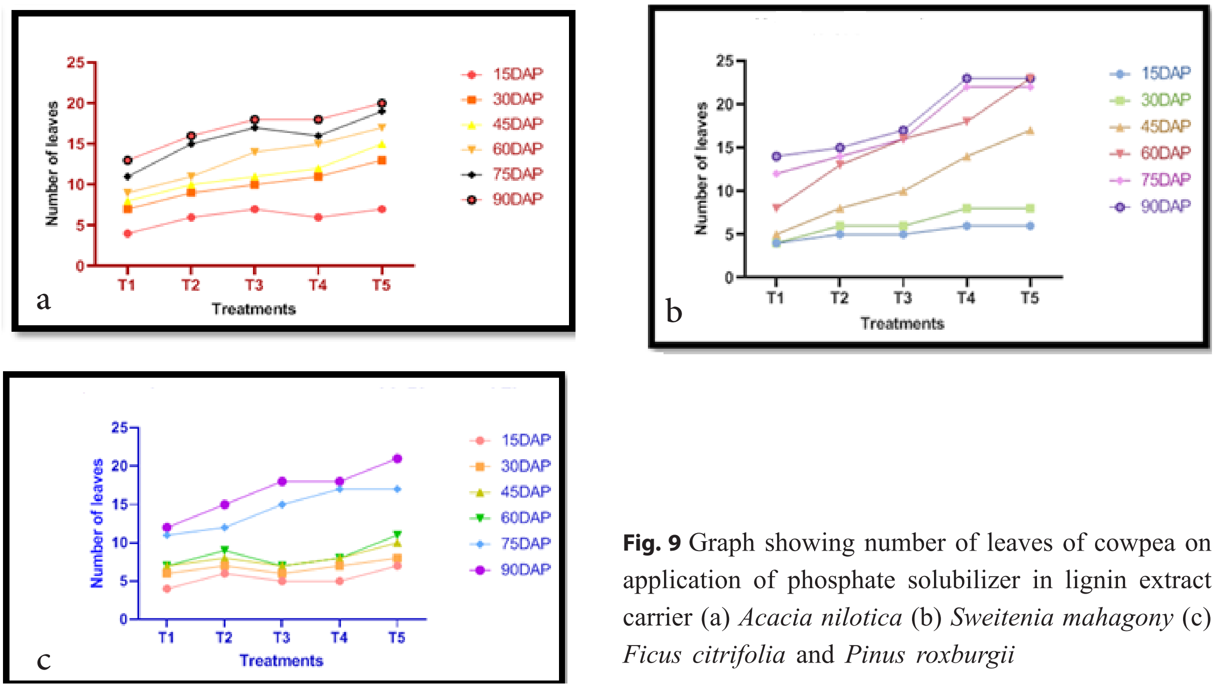

Fig. 9 Graph showing number of leaves of cowpea on application of phosphate solubilizer in lignin extract carrier (a) *Acacia nilotica* (b) *Sweitenia mahagony* (c) *Ficus citrifolia* and *Pinus roxburgii*

Vigna unguiculata (Cowpea) because its application reduced the negative effects of saline water to the biometric, physiological and productive variables of cowpea, aiding in the full development of the crop (da Costa et al. 2020). Hence, in our study we had utilized the lignin extract as carriers to phosphate solubilizers for greater leaf expansion of cowpea plants, increasing the girth efficiency and length of the crop plant.

Conclusion

It was concluded that lignin spent from pulping industry could be utilized as carriers to phosphate solubilizers- *Providencia rettgeri* and *Meyerozyma gullerimondi* isolated from leaf composting units. Here high-quality marketable papers with an average of 172.5 g/m^2 GSM, 1160 m breaking length and 10.43 kg/cm^2 burst

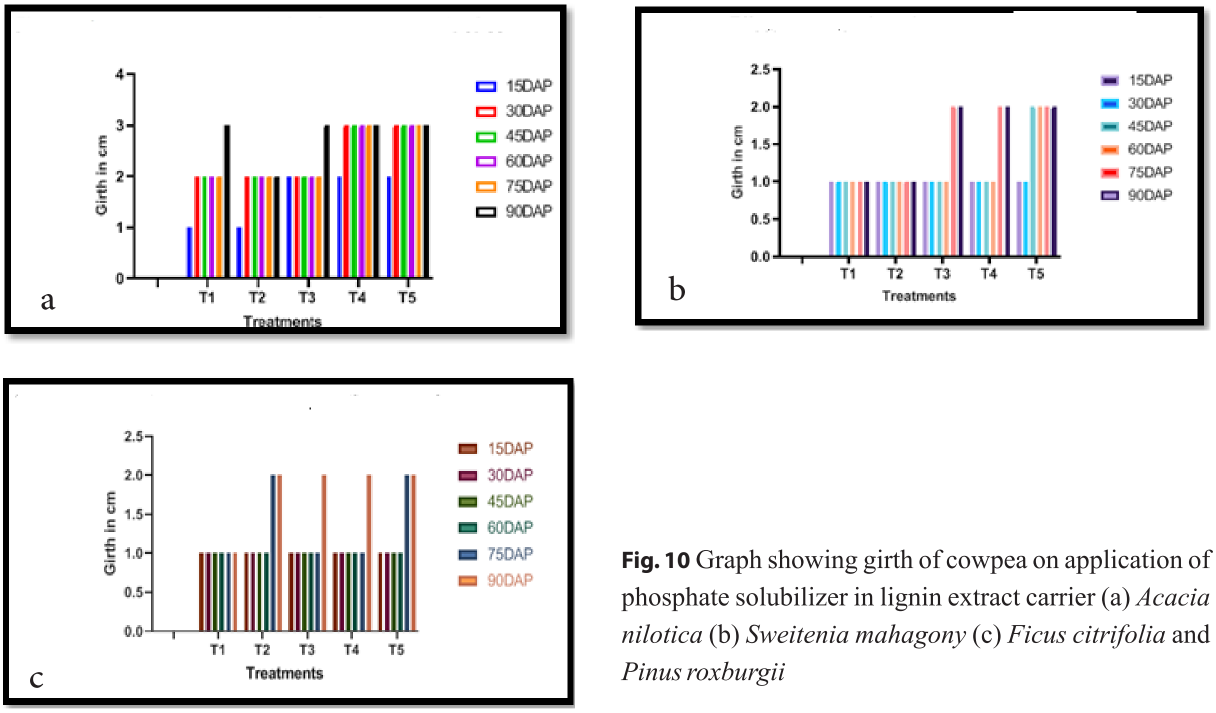

Fig. 10 Graph showing girth of cowpea on application of phosphate solubilizer in lignin extract carrier (a) *Acacia nilotica* (b) *Sweitenia mahagony* (c) *Ficus citrifolia* and *Pinus roxburgii*

factor were developed using cellulose revived from agro-industrial waste biomass like dead-dry leaves of *Ficus citrifolia, Swietenia mahagoni, Pinus roxburgii* and *Musa acuminate.* On recording the biometrics of *Vigna unguiculata* the least value got registered in control (shade plant irrigated with water alone), this also concluded the better fertilizing efficiency of the lignin bio-fertilizers. The nutrient content of potting mixture was reduced after 90 days of planting due to crop removal.

There were several studies on application of paper industry effluent /spent on crops as liquid fertilizer. On considering our study, we had designed a sustainable carrier for bio-fertilizers (phosphate solubilizers) by decomposing environmental waste such as dead/ dry leaves, thereby producing eco-friendly value-added products such as handmade papers. Comparatively, lignin extracts from dry waste leaves after cellulose extraction turns out to be the best carrier for phosphate solubilizers.

Considering the unexploited pedagogy of our research work, the phosphate solubilizers used here could be nano-encapsulated using chitosan/ natural polymers for enhanced uniform release of nutrients to soil. It was already proved that nano-fertilizer increased the nutrient use efficiency, crop yield, and controlled release of nutrients, increased the microbial and plant activity, helped crop to stress tolerance compared to the chemical fertilizers. Many industries now come forward to produce nano-fertilizer in commercial scale. Unfortunately, requirement of nano-fertilizers was much less as compared to the chemical fertilizers. Therefore, if you calculate cost per hectare basis then the nano-fertilizer cost was 2-4 times less than chemical fertilizer. However, the knowledge translation to the farmer was a valid point and industries as well as extension people should take initiative. Government also needed to take initiative to include it under FCO -Fertilizer Control Order so that industries could come forward for larger production that also helped to reduce the cost.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest associated with this study.

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