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BIOFERTILIZER POTENTIAL OF RESIDUAL BIOMASS OF AKK [*CALOTROPIS PROCERA* (AIT.) AIT. F.)]

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Abstract

The biofertilizer potential of residual biomass, derived from two parts that is flowers and leaves of Akk,was investigated in terms of its applications as a substrate for phyto-beneficial bacterial growth and subsequent inorganic phosphate solubilizing agent. The residual biomass was obtained after the extraction of antioxidants from the leaves and flowers of Akk using different solvent systems. The treatment with residual biomass of Akk (RBA) significantly (p<0.05) enhanced the growth of *Enterobacter* sp. Fs-11 and *Rhizobium* sp. E-11 as compared to control (without residual biomass). Maximum microbial growth in terms of optical density (0.92-1.22) was observed for residual biomass sample extracted with aqueous acetone against the control (0.58-0.68). On the other hand, maximum phosphate solubilization (589.27-611.32 µg mL^{-1}) was recorded for aqueous ethanol extracted residual biomass while the minimum (246.31-382.15 µg mL^{-1}) for aqueous acetone extracted residual biomass against the control (576.65µg mL^{-1}). The present study revealed that the tested RBAcan be explored as an effective bio-inoculant to supplement synthetic inorganic phosphate fertilizers. However, some appropriate *in-vitro* assays should be conducted to optimize and standardize the quantity and mesh size of residual biomass prior to use in biofertilizer production as carrier material.

Key words: *Calotropis procera*, Biofertilizer, Phosphate solubilization, Residual biomass, Microbial growth.

Introduction

Locally known as Akk,(*Calotropis procera)*, is a tall, erect and multi-branched plant with characteristic milky secretion (latex) (Khanzada *et al.,* 2008; Meena *et al.,* 2010). This medicinally important plant is wildly distributed in sub-tropical and tropical (hot humid) areas of Asia and Africa. The plant is well known as a potential source of medicinally and physiologically important bioactive substances such as cardiac glycosides, flavonoids, phenolic compounds and terpenoides etc. (Ahmed *et al.,* 2005). The plant is therefore used frequently in the folk/traditional medicine system of several civilizations such as Sudanese, Unani, Arabic and Indian for the prevention and cure of different diseases such as piles, leprosy, liver, spleen, liver and abdomen (Kartikar & Basu, 1994). A milky secretion/latex extracted from *C. procera*fruit and leaves is being used as an abortifacient (Anon., 1950), spasmogenic and carminative and for soothing bronchial asthma (Sharma, 1934). The latex also possesses a range of biological such as antidysentric, antisyphilitic, antirheumatic, antifungal, diaphoretic (Watt & Breyer-Brandwisk, 1962; El-Badwi, 1997), proteolytic (Atal & Sethi, 1961), anticancer (Ajoub & Kingston, 1981; Dhar *et al.,* 1968) and anti-inflammatory activities (Basu & Chaudhary, 1991).

After extraction of different types of bioactives from Akk(*C. procera*) plant parts, the residual biomass of Akk (RBA) is mostly discarded as a waste. As such RBA is rarely investigated for its potential for triggering phyto-beneficial microbial growth and enhancement of their resource acquisition traits. The ultimate success in this area can improve the efficacy of biofertilizers which have potential to influence the soil ecosystem and to produce stimulatory substances for the plants due to the presence of living microorganism (Papendick *et al.,* 2002). Biofertilizers can be diversified depending upon the origin, ecology, physiology, and nutritional supplies of microorganisms and raw materials used for the production. Different types of biofertilizers, depending upon the biochemical composition and requirement of field applications are offered in the market (Anon., 2003; Higa & Parr, 1994).

As a living part of biofertilizers, an increasing interest has been observed towards the nutrient mobilization by rhizospheric bacteria. The use of biofertilizers can be supportive to reduce the energy cost prerequisite for synthetic fertilizers. Use of bio-fertilizer is also important for sustainable agriculture (Shahid *et al.,* 2012; Hardarson & Danso, 1993). Interestingly, different types of root associated microorganisms have potential to enhance the plant growth and yield (Kloepper *et al.,* 1998; Okon, 1985), by improving the supply of mineral nutrients (like Phosphorus, Zinc and Copper) of low mobility in the soil (Cunningham & Kuiack, 1992; Goldstein, 1995; Ikram *et al.,* 1992; Thompson, 1996; Tarafdar & Marschner, 1994; Yasmin *et al*., 2013; Saleem *et al*., 2016).

Keeping in view of the growing uses of bio-based fertilizers as a safer alternative to synthetic ones, this research work was mainly planned to explore utilization

of under-utilized residual biomass of Akk (*Calotropis procera*) for biofertilizer production via investigating its potential as a substrate for phyto-beneficial bacterial growth and subsequent inorganic phosphate solubilizing.

Materials and Methods

Plant sampling: The flowers and leave of Akk(*C. procera* L.)were harvested from the fully mature plants wildly distributed in the surrounding areas of National Institute for Biotechnology and Genetic Engineering (NIBGE) Faisalabad, Pakistan.

A taxonomist (Assistant Professor Dr. Mansoor Hameed) working at the Department of Botany authenticated/ identified the samples. The specimens were cut into small pieces, and dried by placing at ambient conditions in a well ventilated room. The dried plant material was kept in zipped polyethylene bags.

Photograph of AKK [*Calotropis procera* (AIT.) AIT. F.)]

Chemicals and reagents: All the analytical grade chemical and reagents used in the experiments were procured from Sigma Chemical Co. (St. Louis, MO, USA) unless stated otherwise. Microbial cultures, were obtained from National Biotechnology Resource Centre (NBRC), NIBGE, Faisalabad, Pakistan

Collection of residual biomass from plant materials: The residual biomass was obtained after extraction of the ambient-dried plant parts following the procedure as detailed in one of our earlier publication (Ahmad *et al.,* 2011). Briefly, the dried plant material was crushed into a powder having average 80 mesh sizes using a grinder. A 20 g leaf and flower ground material was separately extracted with 200 mL of aqueous methanol (80:20, methanol: water v/v), aqueous ethanol (80:20, ethanol: water v/v) and aqueous acetone (80:20, acetone: water v/v) on a conventional Shaker (Gallenkamp, UK) for about eight hours (normal room temperature). After filtration with Whatman-1 filter paper, the residues were extracted again by fresh solvent and the resulting extractions were combined. The residual biomass, left after extraction, was washed with distilled water thoroughly followed by placing in hot-air oven at 60^{o}C for drying purposes. The dried biomass material was ground and placed in reagent bottles (Pyrex) and sterilized using autoclave machine (TOMY SX-700, Japan) before using for further experiments.

Effect of residual biomass on microbial growth: Using a 250-mL capacity Erlenmeyer flask containing sterilized residual biomass of *C. procera*, the bacterial culture was homogenously grown in LB broth by incubating on orbital shaker (Gallenkamp, UK) at 150 rpm for 24 h. A negative control containing LB plus residual biomass and a positive control containing LB plus beneficial microorganism, *Rhizobium* sp. E-11 and *Enterobactor* sp. Fs-11) were also employed simultaneously. After 24 h of incubation, microbial growth was recorded in terms of optical density (OD_{660}) using double beam spectrophotometer (Camspec M-350, UK).

Effect of residual biomass on the solubilization of phosphate: Sterilized leaves and flowers residual biomass of *C.procera,* mixed with 50 mL of Pikovskaya's broth medium (Pikovskaya, 1948), was placed separately in 250-mL sterilized Erlenmeyer flask along with Fs-11 phosphate solubilizing strain. The flasks were incubated at 28±2^{o}C in an orbital shaker (150 rpm) for 12 days. After incubation, a 40-mL culture volume of bacterial strain was placed in sterilized falcon tubes and centrifuged for ten minutes at 8,000 rpm (4^{o}C). The supernatant was collected in sterilized falcon tubes. Phosphate solubilization was calculated after recording absorbance of phosphomolybdate blue colored complex at (λ=882 nm) using a spectrophotometer (Camspec M-350, UK) (Murphy and Riley, 1962) against the standard phosphorus curve constructed (2-100 ppm). Negative control (Pikovskaya's broth + residual biomass) and positive control (Pikovskaya's broth + strain Fs-11) were also run under the same experimental conditions.

Statistical analysis: Triplicate tests were conducted while the data produced was statistically evaluated by ANOVA, using software STATISTICA 5.5. A probability value of $p \leq 0.05$ was used to consider the significant difference. The results thus generated were given as mean ± SD for triplicate determinations.

Results and Discussion

Effect of residual biomass on microbial inoculants growth: The effect of residual biomass of *C. procera* on plant growth promoting microbial inoculants *Enterobactor* sp. Fs-11and *Rhizobium* sp. E-11 was evaluated. As shown in Tables 1 and 2, the optical density (OD_{660}) data for leaves and flower residual biomass (in the range of 0.69-1.22 and 0.80-1.37, respectively) indicated a better growth of microbial inoculants in the presence of *C. procera* residual biomass relative to the positive control (*Enterobactor* sp.Fs-11and *Rhizobium* sp. E-11). Microbial inoculants *Enterobactor* sp. Fs-11 showed maximum growth (in terms of OD=1.37) with aqueous acetone extracted flowers residual biomass while the minimum growth (OD=1.15) was recorded for aqueous methanol extracted leaves residual biomass relative to the positive control (*Enterobactor* sp. strain Fs-11).

Table 1. Influence of the treatments with residual biomass from flowers and leaves of *C. procera* leaveson the growth of *Enterobactor* sp.strain Fs-11.

Treatments	Microbial growth ($O.D_{660nm}$)	
Residual biomass extracted with	**Leaves**	**Flowers**
Aqueous methanol	$1.15 \pm 0.02^{b}{}_{b}$	1.24 ± 0.03^{b}a
Aqueous ethanol	$1.16\pm 0.03^{b}{}_{a}$	$1.25\pm 0.04^{b}{}_{a}$
Aqueous acetone	$1.22\pm 0.04^{a}{}_{b}$	$1.37\pm 0.03^{a}{}_{a}$
Control	0.68 ± 0.02^{c}	

O.D: Optical density

The data given is represented as mean ± SD of replicate/ three independent tests. The different letters in superscripts in the identical column designate significant (p<0.05) differences of data among three extraction solvents employed. The different letters in subscripts in the identical row designate significant (p<0.05) differences of data between the two plant parts.

Table 2. Influence of the treatments with residual biomass from flowers and leaves of *C. procera* leaves on the growth of *Rhizobium* sp. (E-11).

Treatments	Microbial growth ($O.D_{660nm}$)	
Residual biomass extracted with	**Leaves**	**Flowers**
Aqueous methanol	$0.72\pm 0.02^{ab}{}_{b}$	$0.82 \pm 0.04^{b}{}_{a}$
Aqueous ethanol	$0.69 \pm 0.02^{b}{}_{b}$	$0.80\pm 0.03^{b}{}_{a}$
Aqueous acetone	$0.81 \pm 0.03^{a}{}_{b}$	$0.92\pm 0.04^{a}{}_{a}$
Control	0.58 ± 0.02^{c}	

O.D: Optical density

The data given is represented as mean ± SD of replicate/ three independent tests. The different letters in superscripts in the identical column designate significant (p<0.05) differences of data among three extraction solvents employed. The different letters in subscripts in the identical row designate significant (p<0.05) differences of data between the two plant parts.

Table 3. Influence of the treatments with residual biomass from flowers and leaves of *C.procera* leaves on phosphorus solubilization.

Treatments	Phosphorus solubilization (µg mL^{-1})	
Residual biomass	**Leaves**	**Flowers**
80% Methanol	$453.96\pm 8.09^{b}{}_{b}$	$539.06 \pm 9.74^{c}{}_{a}$
80% Ethanol	$589.27 \pm 9.65^{a}{}_{a}$	$611.32\pm 8.62^{a}{}_{c}$
80% Acetone	$246.31 \pm 4.92^{c}{}_{b}$	$382.15\pm 6.88^{d}{}_{a}$
Control	576.65 ± 8.35^{ab}	576.65 ± 8.34^{b}

The data given is represented as mean ± SD of replicate/ three independent tests. The different letters in superscripts in the identical column designate significant (p<0.05) differences of data among three extraction solvents employed. The different letters in subscripts in the identical row designate significant (p<0.05) differences of data between the two plant parts.

While, another microbial strain *Rhizobium* sp. E-11 had maximum growth (OD=0.915) with flower residual biomass extracted with aqueous acetone and minimum growth (OD =0.687) with residual biomass of leaves extracted with aqueous ethanol as against the positive control (*Rhizobium* sp. E-11). A notable (p<0.05) increase in microbial growth due to the subject treatments as against the control may be linked to the occurrence of some useful biochemicals in the residual biomass of *C. procera* whichenhanced the growth. It has been reported that the soil microorganisms/microbes use plant phenolics released through the roots and thus exert beneficial effects towards better growth and productivity of plants (Daniel & Anderson, 1992). The present work is likely the first study dealing with the investigation of the influence of *C. procera* residual biomass on the growth of microbial inoculants being used in the biofertilizer production; hence it is not possible to as such compare the data of the current analysis with the earlier reports in the literature.

Effect of residual biomass on the solubilization of phosphate: Nutritionally both the phosphorus and nitrogen are regarded as essential/elements required to the plants for their better growth, development and productivity (Wua *et al.,* 2005). A huge quantity of fertilizers is used in agriculture sector every year to sustain and/or enhance the yield and productivity of crops. However, the use of chemical fertilizers on a large scale on crops not only involves high cost but also causes environmental and health problems (Dai *et al.,* 2004). The processes such as fixation of nitrogen (N_2) along with phosphate solubilization are essential and basic characteristics of biofertlizers for improved crop yields. Biofertlizers, mostly contain such types of soil microrganisms which have the ability of nitrogen fixation and phosphate solubilization and thus change unavailable phosphorus form to an available from for plant uptake. The present study also investigated the potential of the tested residual biomass of *C. procera* towards improving phosphorus solubilization capacity of the microorganisms tested. The results in Table 3 depicted that microbial inoculants, when leaves and flower residual biomass was supplemented, mobilized the insoluble form of phosphorus in the range of 246.31-589.27; 382.15-611.32 µg mL^{-1}, respectively. Phosphate solubilizing bacteria (*Enterobactor* sp. FS-11) mobilized maximum amount (611.32 µg mL^{-1}) of insoluble tricalcium-phosphate in Pikivskayas's medium in the presence of flower residual biomass extracted with aqueous ethanol while minimum value of phosphate solubilization (246.31 µg mL^{-1}) was observed with residual biomass extracted with aqueous acetone.

Overall, Pakistani soils are reported to be alkaline in nature, carrying mostly phosphorus in an unavailable form, which cannot be available and taken up by the plants for their growth. Therefore, a suitable level of acidic soil environment is needed in the neighborhood of plants root so as to mobilize insoluble form of phosphorus. With the exception of aqueous ethanol extracted residual biomass derived from flowers and

leaves of *C. procera*, none of the other treatment mobilized/solubilized the insoluble form of phosphorus considerably relative to the positive control (*Enterobactor* sp. Fs-11). This unusual pattern can be supported and explained and linked to the presence of certain biochemical in the specific/related residual biomass which decreased pH of the growth media to an optimum level and provided acidic environment required for phosphorus solubilization. No earlier reports are available on studying the influence of *C. procera* derived residual biomass on phosphorus solubilization, hence, this contribution may serve as a platform to explore phosphorus solubilization potential of the tested under-utilized and agrowaste material and hence a step-forward towards the utilization of natural resources for sustainable agriculture.

Conclusion

It could be concluded form the results of this scientific contribution that the residual biomass of *C. procera* extracted with aqueous ethanol might serve as stimulating agent for beneficial microbial growth and subsequent phosphate solubilization. However, aspects relating to its application as supporting agent in biofertilizers need further studies under green house and field conditions. Furthermore, investigation regarding assessment of economic and environmental impacts, before recommending the uses of the tested biomass as carrier material for biofertilizers, is also recommended.

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