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Effect of calcium salts on growth and plant growth promoting activities of salt tolerant *Azotobacter chroococcum*

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Abstract
Effects of chloride and sulphate salts of calcium on the growth and plant growth promoting activities of salt tolerant *Azotobacter chroococcum* strain H12 were studied. *A. chroococcum* strain H12 was assessed for tolerance to various concentrations of chloride and sulphate salts of calcium. With increase in the concentration of calcium chloride, there was a decrease in the growth, while calcium sulphate had a beneficial effect of the growth of the microbe. Additionally, salts had no effect on osmolytes proline accumulation in the cells while there was an increase in amino acid content at lower concentration of both the salts. Plant growth promoting activities of these cultures under salt stress were also determined. Nitrogen-fixing ability was not influenced by sulphate salt and enhanced in the presence of chloride salt of calcium. Similarly, IAA production was not affected by sulphate salt and was considerably reduced by supplementation with $CaCl_2$. In contrast, there was beneficial effect of low concentration of chloride salt and negative effect of sulphate salts of calcium on P-solubilization ability. The *A. chroococcum* culture was observed to be positive for siderophore production and negative for HCN production. Wherefore, the present study confirmed that strain H12 could survive and carry out plant growth promotion activities under saline condition. This culture has the potential to be developed into bioinoculant for saline soils.

Keywords: *Azotobacter*, salinity tolerance, calcium chloride, calcium sulphate, acetylene reduction activity, P-solubilization, IAA production

Introduction

Agriculture is considered to be one of the most vulnerable sectors to climate-change. Crop production, particularly in tropical regions is facing increasing stresses caused due to natural and anthropogenic factors [1]. Dominant abiotic stresses comprise drought, submergence, low/ high temperature, salinity and acidic conditions, light intensity and nutrient deficiency. Of the total global land area, 64% of the land is affected by drought, 13% by flood, 6% by salinity, 15% soil alkalinity, 9% by mineral imbalance and 57% by cold [2]. Salinity stress is one of the major abiotic stress limiting crop growth and productivity. Generally, lack of sufficient moisture causes soil to become more saline and alkaline [3]. It was estimated that about 20% irrigated land of the world under cultivation is salt affected and 10 million ha per year is destroyed by salt accumulation. Out of the world's 5.2 billion ha of dryland agriculture, nearly 3.6 billion ha of land is affected by moderate to high soil salinization which subsequently, may lead to soil degradation [4]. Reports have been estimated that more than 50% of the arable land would be salinized by the year 2050 [5].

Salinity stress is mainly contributed by the presence of dissolved salts such as NaCl, Na_2SO_4, $MgSO_4$, $CaSO_4$, $MgCl_2$, $CaCl_2$, KCl and Na_2CO_3 which increase soil osmotic pressure and interfere with plant nutrients. Most studies on salt stress and tolerance have been conducted with NaCl, which is generally the most abundant salt. Saline soils also contain high enough concentration of calcium salts [6]. The salt affected soils are unsuitable for crop production and generally are cultivated by marginal farmers who grow salt tolerant crops, do not use any chemical fertilizers and hence do not realize the full yield potential of the crop. Biofertilizers are easily affordable alternative to chemical fertilizers for improving crop yields.

Azotobacter, a free-living rhizobacterium, has been used extensively as a bioinoculant in a wide variety of crops like cereals, fibre crops, oil seed crops, sugarcane, vegetables and fruit crops for improving growth and yield [7]. *Azotobacter* have been observed to possess many plant growth promoting activities [8] such as nitrogen fixation, phosphorous solubilization, production of phytohormones like IAA, gibberellins and cytokinins, vitamins and amino acids. These also produce antibiotics and siderophores which have inhibitory action towards phytopathogens. However, most of the *Azotobacter* strains used as bioinoculants are effective only in normal soils and fail if used under saline conditions.

Under saline condition, survivability of microorganisms is drastically affected and many of the vital bacterial plant growth promoting activities, such as nitrogen fixation and phytohormone production are inhibited [9]. For use as bioinoculant under salt stress conditions it is imperative to isolate and screen for salt tolerant *Azotobacter* strains and use the most efficient strain to develop bioinoculants for saline soils. With this objective, in the present investigation, *Azotobacter* sp. were isolated and screened for its salt tolerance. Effect of chloride and sulphate salts on growth, osmolyte pool and plant growth promoting activities of these strains were also determined.

Materials and methods

Bacterial culture and maintenance

Salt tolerant *Azotobacter chroococcum* strain H12, isolated from saline soil samples collected from salt affected areas of Hissar, Haryana, India, was used in the present study (Paul *et al*., 2014) and was maintained on Jensen's N-free medium [10].

Effect on bacterial growth

A. chroococcum strain H12 was grown in Jensen's N-free broths supplemented with 0.1M to 1.7M $CaCl_2$, 0.1M to 0.3M $CaSO_4$. Jensen's N-free broth without additional salt served as control. Broth was inoculated with 2% inoculum and tubes were incubated in an orbital shaker at 28±2°C for 5 days. Three replications of each treatment were maintained. Growth on different salts was determined by protein estimation by Lowry's method [11].

Effect of salts on the osmolyte pool

Effect of different concentrations of chloride and sodium salts of calcium on the osmolytes proline and amino acid pool of *A. chroococcum* strain H12 released by cell lysis were studied. Salt concentrations of 0.4M and 1.7M $CaCl_2$, 0.2M and 0.3M $CaSO_4$ were used in this study. *A. chroococcum* culture was grown in Jensen's broth supplemented with various concentrations of salts, incubated at 28±2 °C for 6 days on orbital shaker. These were centrifuged at 15000 rpm for 10 min for collecting cell pellets and lysis was done by using 20% TCA. Collected lysates were used for the analysis. Amino acid content was estimated by the method of Chen *et al* (2006) [12]. One ml of 0.1 M sodium acetate acetic acid buffer pH 4.3, 1 ml of Ninhydrin reagent (5% Ninhydrin in ethanol) and 1ml of sample supernatant was mixed, vortexed and immersed in a hot water bath (95^0C) for 15 min. Observations were taken at 570nm using spectrophotometer after cooling the samples at room temperature. Proline was determined by the method of Bates *et al* (1997) [13]. One ml of the sample was mixed with 2ml Ninhydrin reagent and was boiled for one hour in water bath. The color was then extracted with the help of 2ml toluene and the observations were taken at 520nm.

Salts on plant growth promoting activities

Plant growth promoting activities such as nitrogen fixation, IAA production, P-solubilization, HCN and siderophore production of *A. chroococcum* strain H12 at different concentrations of chloride (0.4M and 1.7M) and sulphate (0.2M and 0.3M) salts of calcium were determined. Three replications per treatment and appropriate uninoculated controls were maintained.

Nitrogen fixation

Nitrogen fixing ability of *A. chroococcum* strain H12 was determined by the acetylene reduction activity (ARA) method [14]. *A. chroococcum* strain H12 was grown on Jensen's N-free slants supplemented with different concentrations of calcium salts. After three days of incubation in an incubator at 28±2°C, 10% of the airspace in the test tube was replaced with acetylene and the slants were incubated for another 24 hrs. After that 1 ml of gas sample from the test tubes was injected into injector port of Gas chromatograph (Nucon 5765 model) fitted with FID detector and Porapak N column. Nitrogen fixing ability was expressed as nmoles of ethylene produced/mg protein/hr.

IAA production

A. chroococcum strain H12 was tested for the production of IAA by the method of Hartmann (1983) [15]. The cultures were grown in Jensen's N-free broth media supplemented with salts and 1mM tryptophan. Inoculated broth was incubated on an orbital shaker at 28 ± 2ºC for 5 days. Supernatant was collected by centrifuged at 10,000g for 10 min. One ml of supernatant was added with 4 ml of Salkowski's reagent and developed pink colour was measured at 535 nm using Perkin Elmer spectrophotometer. IAA production by the cultures was expressed as µg IAA produced/mg protein.

P- Solubilization

Pikovyskaya's broth supplemented with different concentrations of $CaCl_2$ and $CaSO_4$, were used for the determination of phosphorous solubilization. The tubes were inoculated with 2% of the desired culture suspension and allowed to grow for 7 days at 28±2 ºC on an orbital shaker. The solution was then transferred to the centrifuge tubes and centrifuged at 8000 rpm for 10 minutes. Phosphate solubilized in the supernatant was determined by the method of Jackson (1967) [16]. In each case 1 ml of the clear supernatant was added with ten ml of Chloromolybidic acid, 5 drops of stannous chloride and volume was made up to 50 ml. Observations were taken immediately at 660 nm using Perkin Elmer spectrophotometer, model Lambda E2201. The quantity of phosphate solubilized was expressed as µg P solubilized/mg protein.

HCN and siderophore production

A. chroococcum strain H12 was streaked on King's B agar plates amended with 4.4 g/l of glycine and supplemented with different concentrations of chloride and sulphate salts of calcium. HCN production by culture was detected by the method of Bakker and Schipper (1987) [17]. CAS agar media was supplemented with different concentrations of sodium and chloride salts of calcium. Siderophore production was detected by using Chrome azurol assay (CAS) developed by Schwyn and Neilands (1987) [18].

Statistical analysis

Data generated were subjected to one way analysis of variance (ANOVA) using the statistical package WASSP 2.0 from ICARGOA. The means in all these analysis were separated using the least significant difference test at $P < 0.05$.

Results and Discussion

In nature, microorganisms inhabiting or exposed to extreme conditions have tolerance or develop adaptability to survive in those extreme conditions. The present investigation was aimed to assess the effect of calcium salts on growth and plant growth promoting activities of salt tolerant *A. chroococcum* strain H12, isolated from salt affected areas of Aligarh, Uttar Pradesh and Hissar, Haryana [19].

Effect on growth

Effect on the growth of *A. chroococcum* strain H12 in terms of µg protein produced per ml of broth supplemented with different concentration of salts were determined (Figure 1). Increasing concentration of chloride salt of calcium, led to decrease in the growth compared to control. Lowest growth was obtained in presence of 0.8 M $CaCl_2$. However, with increase in the

concentration of calcium sulphate salt, there was an increase in the growth. Only in presence of 0.3 M $CaSO_4$, a decrease in growth as compared to 0.25 M concentration was observed, however, it was still higher than that observed under control condition. Highest growth was obtained in the presence of 0.25 M $CaSO_4$. These observations confirm the calcium salt tolerance ability of strain H12.

Effect of salts on metabolite pool of A. chroococcum

Influence of salts on the metabolite pool of *A. chroococcum* strain H12 was determined (Figure 2). Salt adversely affects bacterial growth and there is decrease in growth of a microbe with increase in salt in the growth medium [20, 21]. Similarly in our study also protein and amino acid content produced by the culture was affected with increasing calcium chloride concentration. There was an increase in protein pool with increase in concentration of calcium salts. Only in presence of 0.8 M $CaCl_2$, protein pool was similar to control. In presence of 0.3 M $CaSO_4$ highest protein pool was obtained. At lower concentration of chloride and sulphate salts of calcium, there was an increase in amino acid pool but at higher concentration of these salts, there was decrease in amino acid content. Highest amino acid content was obtained in the presence of 0.2 M $CaSO_4$.Similar increase in amino acid content in presence of salinity were also reported in *Mesorhizobium ciceri* [22]. Proline augmentation induces adjustment of cell osmotic potential and cell adaptation under osmotic stress condition [23, 24]. However, proline production by H12 was observed to be either reduced or no variation in different salt concentrations compared with control. These results showed proline probably did not play an important role in salt stress tolerance of *A. chroococcum* strain H12.

Salts on PGPR activities

PGPR activities such as nitrogen fixation, IAA production and P-solubilization by *A. chroococcum* strain H12 in medium supplemented with different concentrations of salts were determined (Table 1). There was an increase in ARA activity in the presence of calcium chloride. Highest ARA activity was observed in Jensen's medium supplemented with 0.4 M $CaCl_2$. Although, slight decrease in ARA activity of *A. chroococcum* strain H12 in the presence of calcium sulphate salts was observed, it was at par with control treatment. Thus, the nitrogen fixing ability of this culture was not negatively affected by the presence of high concentrations of salt. Diazotrophs are known to benefit plants under salinity stress by increasing nitrogen availability to plants [25].

Increasing concentration of salts led to decrease in the production of IAA. Broth supplemented with $CaCl_2$, drastically reduced the production of IAA. IAA production by *A. chroococcum* strain H12 was more or less similar with control in broths supplemented with $CaSO_4$. Several stages of plant growth including cell division, elongation, tissue differentiation and apical dominance etc are known to be controlled by Indole-3-acetic acid known as auxin. PGPR strains were reported to synthesize IAA, which aid in plant growth promotion. Osmotolerant cultures of *Azotobacter chroococcum* and *Azotobacter vinelandii* were observed to produce IAA under saline condition, which improved the growth of wheat plants [26].

P-solubilization ability is one of the important plant growth promoting activities of microorganisms, which plays an effective role in the accumulation and transformation of phosphate in plant roots. It has been reported that phosphate solubilizing bacterial inoculum increased the growth and yield of crops such as wheat and maize [27, 28]. Phosphate solubilizing ability tends to decrease with increasing salt concentrations [29]. Similarly P-solubilization by *A. chroococcum* strain H12 was affected by the supplementation of sulphate salt of calcium. Highest P-solubilization by *A. chroococcum* strain H12 compared to control was observed in 0.4M $CaCl_2$ treatment.

Effect of salt on biocontrol activities of *A. chroococcum* strain H12, such as HCN and siderophore production, was also determined (Figure 3). HCN producing PGPRs mainly serve as biocontrol agents by protecting the plants from diseases and preventing growth of weeds. Production of HCN was observed in H12 culture. Numerous bacterial cultures release colorless or fluorescent pigment siderophores to the medium when grown under iron limiting conditions. Siderophores are powerful and selective low molecular weight iron chelators whose biosynthesis is repressed by Fe (III). Good amount of siderophore production was observed in *A. chroococcum* strain H12. Presence of clear halo around the bacterial colony confirmed that this culture was positive for siderophore production.

Table 1: Effect of different concentrations of chloride and sulphate salts of calcium on plant growth promoting activities

Treatments	IAA production (µg IAA produced/ mg protein)	P solubilized (mg P solubilized/ mg protein)	ARA activity (n moles of ethylene produced/mg protein)
Control	36.79 ± a	474.93 ± 14.24 b	411.15 ± 10.11 b
0.4M $CaCl_2$	1.7 ± 0 b	1470.45 ± 35.03 a	1328 ± 58.21 a
1.7M $CaCl_2$	1.7 ± 0 b	251.1 ± 5.3 c	1244.99 ± 61.74 a
0.2M $CaSO_4$	35.12 ± 1.54 a	278.14 ± 37.76 c	226.72 ± 16.02 b
0.3M $CaSO_4$	31.36 ± 2.52 a	210 ± 29.98 c	348.36 ± 14.43 b

Values are means of three replications.

Means followed by a common letter in a column are not significantly different at $p < 0.05$.

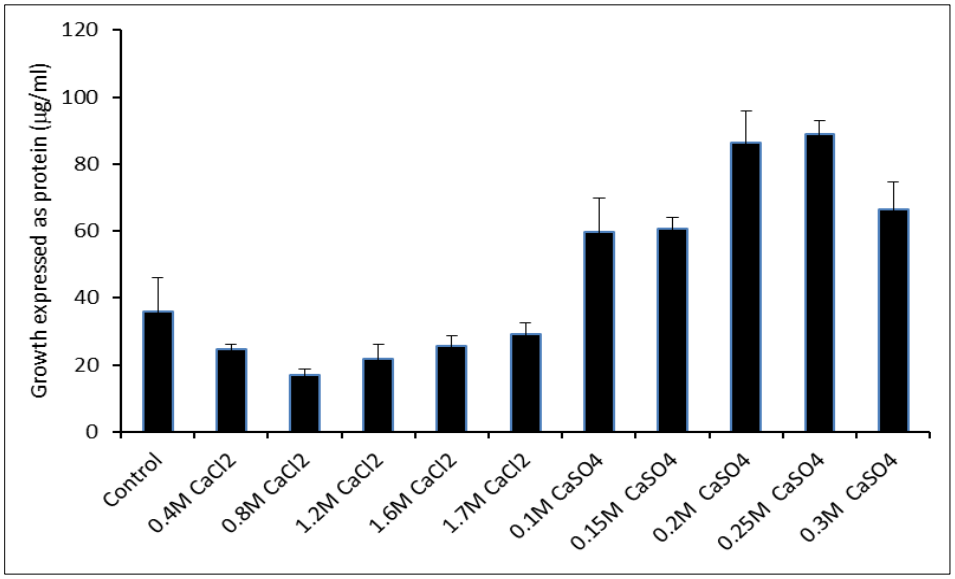
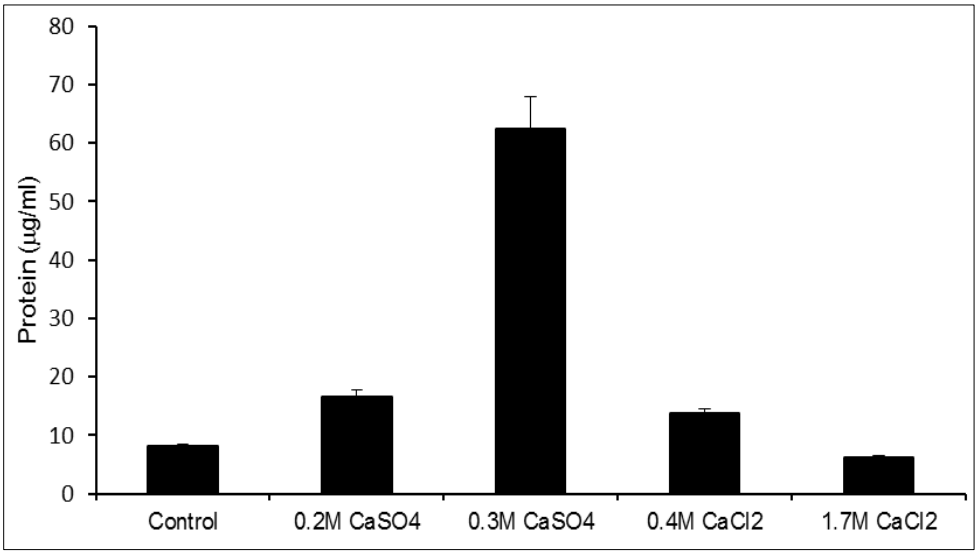
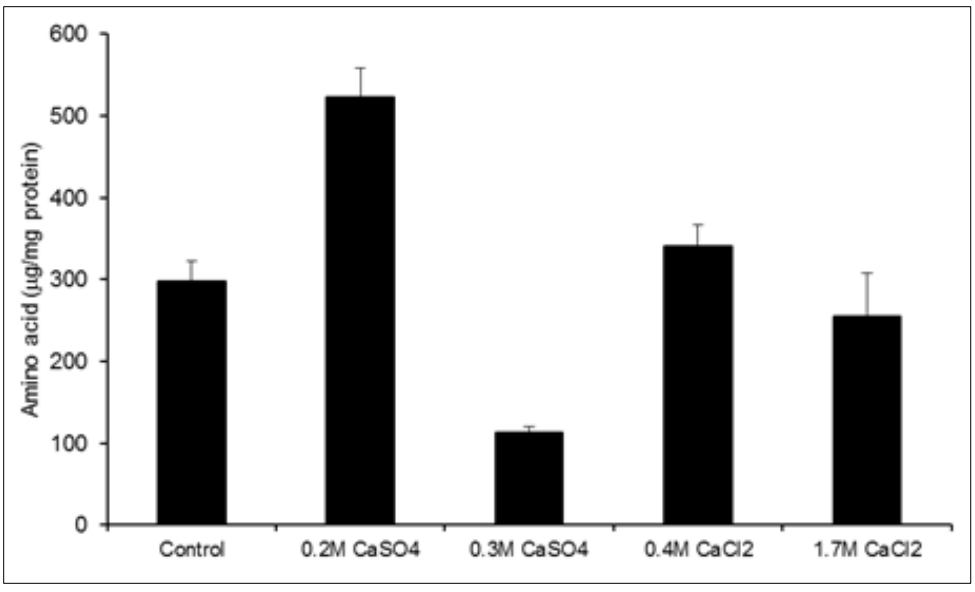


Fig 1: Influence of a) calcium chloride b) calcium sulphate at different concentrations on the growth of *Azotobacter chroococcum* strain H12. Error bars indicate the standard deviation of the means from three replicates; Histograms with different letters are statistically different at $p < 0.05$.





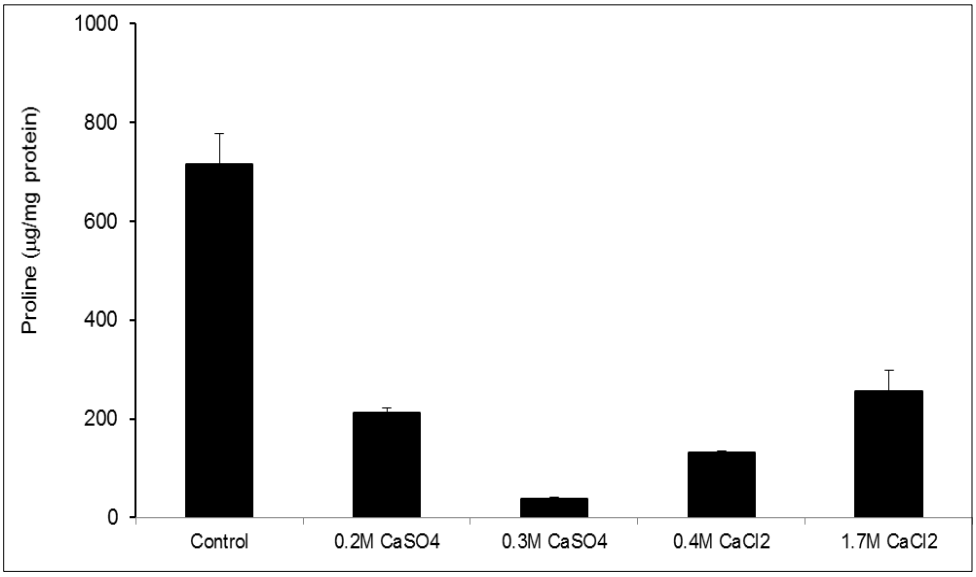


Fig 2: Effect of chloride and sulphate salts of calcium on the metabolite pool of *Azotobacter chroococcum* strain H12 released by cell lysis. A) Protein pool B) Amino acid pool C) Proline pool. Error bars indicate the standard deviation of the means from three replicates; Histograms with different letters are statistically different at $p < 0.05$.

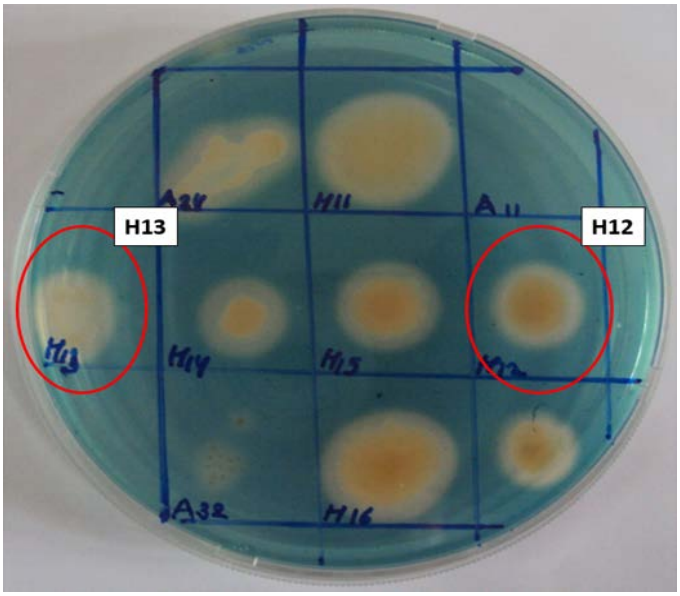


Fig 3: Siderophore production by *Azotobacter chroococcum* strain H12 Clear zone around the colonies of H12 indicates the production of siderophore.

Conclusion

A. chroococcum culture H12, isolated from saline soils showed tolerance to chloride and sulphate salts of calcium, which are prominent components of saline soils. Even though, high salt concentrations affected growth and plant growth promoting activities of these cultures, these negative effects were not that severe. To our knowledge, this is the first report dealing with *A. chroococcum* tolerance against chloride and sulphate salts of calcium. The culture not only survived but also was able to carry out PGP activities under saline conditions. Comprehensive results showed that the *A. chroococcum* strain H12 selected in this study could be employed as bioinoculants under saline conditions. Hence, further studies on their effect on crop growth need to be carried out to get great economic and environmental benefits.

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