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Pesticide Tolerance *Azotobacter* sp., from Crop Field

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ABSTRACT: Use of chemical fertilizer is responsible for the loss of soil texture, soil fertility and also food nutrition. But, farmers couldn't cultivate without chemical fertilizers and pesticides because of the presence of huge pests in the environment. In this point, only pesticide tolerant nitrogen fixers are very much essential to take the challenges to return the soil texture and fertility. In relation to the current context, one *Azotobacter* sp., shown to resist the maximum percent of three chemical pesticides, CP α, CP β, CP γ, one bactericide and one bio-pesticide, Neem seed oil in presence of fertilizers, UREA, DAP, NPK (10:26:26), MOP, SSP, NPK (15:15:15) which ultimately reflected in crop production by supplying fix nitrogen to the soil. A critical work has been done to find out a pesticide resistance strain, *Azotobacter* sp., which ultimately will solve a long waited cultivator's problem for crop improvement.

Keywords: *Azotobacter*, Chemical fertilizer, Chemical pesticide, Chemical bactericide, Bio-pesticide.

INTRODUCTION

Nowadays, chemical fertilizers, chemical pesticides and chemical bactericides are used on a large scale in agricultural fields for higher crop yield (Wang *et al.*, 2020). These chemical pesticides and chemical bactericides are used in agriculture fields to protect plants against different types of harmful pests, insects and bacteria (Özkara *et al.*, 2016). But, the use of these chemicals in large amounts are hampering soil fertility, soil ecosystem, soil microbe interaction and soil organic matter in the crop field. Therefore, these chemicals antagonistically influence the *Azotobacter* population in soil (Chennappa *et al.*, 2014). Many studies have shown the minimum amount of chemical fertilizer, chemical pesticides and bacteriocide did not affect the Agricultural environment (Fanelli, 2020); (Dar *et al.*, 2019) but excessive and prolonged usage of these chemical-containing compounds may affect soil fertility and soil microbial population. Chemical fertilizers should be replaced with biofertilizers and chemical pesticides with biopesticides to improve plant growth as well as to maintain environment-friendly soil properties for sustainable crop production (Bhaduri *et al*., 2016).

Biofertilizers can maintain soil fertility without any adverse effects on the environment (Suhag, 2016). Instead of chemical fertilizers, biofertilizers are beneficial for normal soil microbial populations. Biofertilizers are used in the agriculture field to maintain soil fertility and ecological balance (Vijayeswarudu and Singh 2020). The nutrition values of biofertilizers are equal to chemical fertilizers and the former could be used for higher crop cultivation (Medhi *et al.*, 2007). Among different types of biofertilizers, *Azotobacter* containing one is important for asymbiotic plant growth and high crop yield. Another important criterion of *Azotobacter* biofertilizers is low priced material (Gomare *et al.*, 2013). *Azotobacter* can tolerate low amounts of chemical fertilizers, chemical pesticides, and chemical bactericides (Gurikar *et al.*, 2016). Metabolic functions of *Azotobacter* are not affected by the presence of different pesticides and they are able to grow and survive in these harsh conditions. *Azotobacter*maintains the ecological balance with an effective impact on the biodegradation of the pollutants (Chennappa *et al.*, 2013). *Azotobacter* is an important member of the soil that increases plant growth and soil fertility (Manoj *et al.*, 2022). *Azotobacter* is now used as a biofertilizer to maintain soil fertility and increase crop production in non-symbiotic process (Aasfar *et al.*, 2021).

Bio-pesticide is very cost effective, less active and environmentally friendly compared to chemical pesticides and equally effective against many harmful insects, pests, fungi, weeds, bacteria and viruses (Kawalekar, 2013 ; Masoodi *et al.*, 2022). Biopesticides don't damage the molecular path of the plant root (Chauhan and Varshneya 2012). Plant-based biopesticide such as Neem seed oil is the most important in the agricultural sector to control a large number of insects, pests, nematodes, arthropods, fungi, virus and snails (Sharma *et al.*, 2021). Neem seed contains Azadirachtin, a tetranortriterpenoid component that helps to prevent pests by inhibiting the metamorphosis of insects (Dhir, 2017).

MATERIALS AND METHODS

Collection of soil sample. The soil sample was collected in a sterile container from the agricultural field of Talgachhi, Malda district (25° 38′ 62″N 87° 83′ 93″E), West Bengal, India and brought to the laboratory in aseptic condition.

Isolation of *Azotobacter* sp. One gram soil sample was mixed with 10 ml autoclaved distilled water. The soil suspension was allowed to settle for some time for the precipitation of insoluble particles in the bottom. The upper clear suspension was poured in nitrogen free burk's agar medium (Magnesium sulphate 0.200 Gms / Litre, Dipotassium hydrogen phosphate 0.800 Gms / Litre, Potassium dihydrogen phosphate 0.200 Gms / Litre, Calcium sulphate 0.130 Gms / Litre, Iron (III) Chloride 0.00145 Gms / Litre, Sodium molybdate 0.000253 Gms / Litre, Sucrose 20.000 Gms / Litre, agar 20 Gms / Litre,) for the isolation of free living nitrogen fixing soil bacteria. The plate was incubated at 30 ºC for overnight. Among several colonies, some fast-growing, gummy, round, creamy or slidely colored bacterial colony was selected for further study. The bacterial culture was purified by repeated streaking on a nitrogen-free burk's agar plate.

***Azotobacter* Characterization.** Isolated colonies were tested for carbohydrate, amino acid, protein utilization with other tests, gram stain, nitrate reductase, catalase test in this study. The authenticated and published procedures form Bergey's manual of Systematic Bacteriology (1994) and also consulting with the published procedure of different author work. All the chemicals were used from Hi-media, Loba, SRL and Sigma chemical company. The results were recorded very carefully using triplicate experiments.

Growth characteristics of the isolate in different supplemented materials. Growth (OD 660) of the isolate was measured in nitrogen-free burks broth supplemented with six types of chemical fertilizers (UREA, DAP, MOP, SSP, and two types of NPK), three types of chemical pesticides (CP α-Fighter 500, CP β- Tiger 10 and CP γ- Superkiller 10), one chemical bactericide (Bacterinash 200) and one bio-pesticide (Neem seed oil). Each chemical fertilizer was used in different concentrations such as 0.5%, 0.7%, 0.9%, and 1.1% in triplicate. Similarly, 0.1%, 0.2%, 0.3%, 0.4% concentrations of chemical pesticides; 0.1%, 0.2%, 0.3%, 0.4% concentrations of chemical bactericide and 0.3%, 0.6%, 0.9%, 1.2% concentrations of bio-pesticide were added to the medium. The composition of all supplement materials has been included in Tables (1-4).

Tolerance against chemical fertilizers, chemical pesticides, chemical bactericides and bio-pesticide of the isolate. The isolate showed growth (tolerance activities) in five concentrations of different chemical fertilizers (0.5%, 0.7%, 0.9%, 1.1%, 1.3%), chemical pesticides (0.1%, 0.2%, 0.3%, 0.4%, 0.5%), chemical bactericide (0.1%, 0.2%, 0.3%, 0.4%, 0.5%) and bio-pesticide (0.3%, 0.6%, 0.9%, 1.2%, 1.5%, 1.8%) containing nitrogen free burk's medium (Table (5, 6, 7, and 8)).

Statistical Data analysis. All statistical data of this study were analyzed by Microsoft Excel. This study reveals to statistical data including mean, median and standard deviation was calculated by analysis of variance (ANOVA) Statistical significance was indicated at a probability level of P>0.05.

RESULTS AND DISCUSSION

Based on the experimental results the isolated bacterial strain is *Azotobacter* sp., as per Bergey's Manuals Determine of Bacteriology (1984) and Bergey's Manuals Systematic of Bacteriology (1994) Table-9& 10.

Growth of the isolate in different chemical fertilizers. The isolate of this work showed the highest growth in 0.9% UREA and NPK (10:26:26), in 0.7 % MOP, SSP and NPK (15:15:15) compared with the control. The growth was reduced remarkably above the said concentration (Figure -1). Interestingly the highest bacterial growth was found in DAP with a concentration of 1.1% only. The bacterial growth was hampered with other low and high concentrations of different concentrations of fertilizers tested so far in this study.

Growth of the isolate in different chemical pesticides. The isolate showed growth in different concentrations of chemical pesticides, CP α (Fighter 500), CP β (Tiger 10) and CP γ (superkillar 10) as supplemented in the growth medium used in the present study. The bacterium was able to tolerate up to 0.4% of CP α, CP β and CP γ though the Growth rate was reduced in all concentrations (0.1% to 0.4%) of CP α, CP β and CP γ in comparison with the respective control (Fig. 2A, B, C)). Thus, the bacterium can tolerate high concentrations of the pesticide, though growth was reduced.

Growth of the isolate in chemical bactericide. The isolate showed positive growth in all concentrations of Chemical Bactericide (Bacterinash 200) (0.1 to 0.4%) as supplemented in the medium. The bacterium had similar growth characteristics as above (Fig. 2D).

Growth of the isolate in bio-pesticide. In this experiment, the isolate showed growth at different (0.6%, 0.9%. 1.2%, 1.5%) concentrations of Neem seed oil (as a Bio-pesticide-BP) supplemented medium. The bacterium showed less growth in all concentrations (0.6% to 1.5%) of Neem seed oil as compared to the control (Fig. 2E). As per the study result, the bacterium could tolerate Neem seed oil up to 1.5% concentration.

Growth of the isolate in different chemical fertilizers with chemical pesticides (CP α, β, γ)

CP α- Fighter 500 experiments. The isolated strain can tolerate upto 0.4% CP α pesticide concentration in the presence of 0.9% UREA, 0.9% NPK (10:26:26), and 0.7% MOP as shown in Figure 3. The tolerance level of this strain is only 0.2% even with the highest nitrogen fertilizer supplement either by DAP 1.1% or NPK (15:15:15) with 0.7% as shown in Fig. 3. This strain showed zero tolerance with CP α chemical pesticide even with the highest supplement of nitrogen fertilizer SSP with 0.7% concentration Fig. 3. So this strain showed the highest tolerance (0.4%) of chemical pesticides in the presence of the low concentration of 0.7% of MOP in comparison to other chemical fertilizer DAP, UREA, NPK (10:26:26), SSP and NPK (15:15:15) in this study.

CP β- Fighter 10 experiments. The isolate of this work can tolerate only 0.1% CP β chemical pesticide by in vitro culture study even with the highest application of different nitrogen fertilizers, UREA, DAP, NPK (10:26:26), MOP, NPK (15:15:15) and SSP with a maximum concentration of 1.1% DAP, nitrogen fertilizer Fig. 4.

CP γ- superkillar 10 experiments. The potent strain of this study can tolerate only 0.1% CP γ chemical pesticide in the presence of a higher amount of nitrogen fertilizer, UREA (0.9%) and DAP (1.1%) supplemented medium as shown in figure 5. This isolate showed no tolerance in CP γ chemical pesticide even with the highest nitrogen supplement fertilizers, 0.9% of NPK (10:26:26), 0.7% of MOP, SSP and NPK (15:15:15) as shown in Fig. 5.

Growth of the isolate in different chemical fertilizers with chemical bactericide (CB). In the case of chemical bactericide, the strain of this work can tolerate 0.1% to 0.3% in the presence of SSP, MOP, NPK (151515), DAP, UREA, and NPK (102626) respectively according to Fig. 6.

Growth of the isolate in different chemical fertilizers with bio-pesticide (BP-Neem seed oil). In case of bio-pesticide, the strain of this work can tolerate 0.6% to 0.9% of pesticide in the presence of 0.9% UREA, 1.1% DAP, 0.9% NPK (10:26:26) and MOP in relation to survival nitrogen-fixing bacteria. The works think that the killing effect of bio-pesticide on this strain was not withdrawn even in the presence of the high amount of chemical nitrogen fertilizers, 0.7% SSP and 0.7% NPK (15:15:15) in relation to the growth of said bacterial strain as shown in Fig. 7.

Isolated bacterial strain is a free-living aerobic nitrogen fixer that can grow very fast in the nitrogen-free burk's media where it attends maximum growth after 10 hours of fermentation time without requiring any kind of external chemical nitrogen fertilizers in the media. But in the presence of different chemical pesticides, CP α, CP β, CP γ , CB (chemical bactericide), and neem seed oil (bio-pesticide), the growth of the bacterial strain was severely hampered which means the nitrogen fixation was fully or partially blocked artificially by these chemicals uncontrolled use in the cultivated land by the farmers (Peoples *et al*., 1989; Vijaykumar *et al*., 2021). Alternatively, farmers have no way other than the use strong pesticides to control the resistant pest to meet up the food crisis of the large society (Perkins, 2012; Mishra *et al*., 2018). Farmers only aim to produce huge amounts of food for society without considering the effect of strong pesticides not only on plants but also on the human system (Verma *et al.,* 2022). Again a huge use of chemical fertilizers damages not only the soil bacterial populations but also simultaneous damages of soil texture and fertility (Pahalvi *et al.,* 2021). As a result, human beings are suffering a lot from different aspects including serious health problems in the present society. This work aims at a controlled balanced way of using both pesticides with a maximum tolerable concentration of the bacterial strain. Supplement with a low amount of chemical fertilizers to write the nitrogen-fixing ability of the strain which may ultimately be used for the production of bio-fertilizer (Naher *et al.,* 2016). So, in a balanced way, farming is the only alternative to sustain in the present society excepting the scientific development in the world.

Table 1: Compositions of chemical fertilizers (UREA, DAP, MOP, 10:26:26, SSP & 15:15:15).

Chemical Fertilizers (CFs)	Composition
UREA	Nitrogen-46%
DAP	Nitrogen-18%, P-46%
MOP	Muriate of potash-60%
NPK (10:26:26)	Nitrogen-10%, Phosphorus-26%, Potassium (K)-26%
SSP	Single super Phosphate-16%
NPK (15:15:15)	Nitrogen-15%, Phosphorus -15%, Potassium (K)-15%

Table 2: Compositions of chemical pesticides (CP α, CP β, CP γ).

Chemical Pesticide	Composition	Target site	Effective against
CP α:-Fighter 505 (ISO certified from 2015, No: 9001, R.P.C AGRO INDUSTRIES, West Bengal, India)	Chlorpyriphos- 50% w/w, Cypermethrin- 5% w/w, Adjuvants- 45% w/w,	Leaflet	Aphid, Jassid, Jassid, Thrips, Whitefly, Spodoptera, litura, etc. of cotton, paddy, vegetable, etc.
CP β:-Tiger $_{10}$ (ISO certified from 2008, No: 9001, R.P.C AGRO INDUSTRIES, West Bengal, India)	Cypermethrin- 10% w/w, Adjuvants- 90% w/w,	Leaflet	Fruit borer, fruit & shoot fly, Bihar hairy caterpillar of cereal, vegetable, oil seeds, etc.
CP γ:-Superkiller-10 (ISO certified from 2008, No: 9001, Dhanuka Agritech ltd., Gujarat, India)	Cypermethrin- 10% w/w, Adjuvants- Q.S (Quantitative Study in 100% w/w).	Leaflet	Vegetable

Table 3: Compositions of chemical bactericide (CB) -bacterinash 200.

Chemical Bacteriocide (CB)	Composition	Target site	Effective against	Precaution
CB*:-Bactinash-200 (ISO certified, No: 9001-14001)	2 Bromo-2-nitropropan-1,3-diol-95% w/w and Adjuvants-5% w/w.	leaflet	leaf blight, black arm disease, seeding blight, angular leaf spot, citus canker for cotton, citus, paddy, chillies, betelvine, tomato, banana, grape, vegetables, potato, flowers and fruit crops.	Avoid direct contact with skin and eyes

Table 4: Compositions of bio-pesticide (BP) of neem seed oil.

Bio Pesticide (BP)	Composition	Target site	Effective against	Precaution
BP*:-Neem seed oil (HiMEDIA, REF: RM6541-100G, CAS No: 8002-65-1)	Azadirachtin is the most well-known and studied triterpenoid in neem oil. Nimbin is another triterpenoid that has been credited with some of neem oil's properties as an antiseptic, antifungal, antipyretic and antihist amine.	Leaflet	Mealybugs, beet armyworms, aphids, the cabbage worm, thrips, whiteflies, mites, fungus gnats, beetles, moth larvae, mushroom flies, leaf miners, caterpillars, locusts, nematodes and the Japanese beetle.	Avoid direct contact with skin and eyes

Table 5: Growth of *Azotobacter* in N_2-free Burk's medium with different concentrations of chemical fertilizers (CFs).

Chemical Fertilizers (CFs)	Different concentrations of chemical fertilizers (CFs) used				
	0.50%	0.70%	0.90%	1.10%	1.30%
UREA	+ +	+ + +	+ + + +	+ +	+
DAP	+	+ + +	+ + +	+ + + +	-
NPK (10:26:26)	+ +	+ +	+ + +	+	-
MOP	+ +	+ +	+ + +	+	-
NPK (15:15:15)	+	+ + +	+	-	-
SSP	+ +	+ + +	+	-	-

++++ (Excellent), +++ (Very good), ++ (Good), + (Seen), - (Not seen)

Table 6: Growth of *Azotobacter* in N_2-free Burk's medium with different concentrations of chemical pesticides (CP α, CP β, CP γ).

Chemical Pesticides (CP)	Different concentrations of chemical pesticides (CP α, CP β, CP γ)used				
	0.10%	0.20%	0.30%	0.40%	0.50%
CP α *	+ + +	+ + +	+ + +	+ +	-
CP β *	+ + +	+ + +	+ + +	+	-
CP γ *	+ + +	+ + +	+ + +	+ +	-

CP α *:-Fighter 505, CP β *:-Tiger $_{10,}$ CP γ *:-Superkiller-10;   +++ (Very good), ++ (Good), + (Seen), - (Not seen)

Table 7: Growth of *Azotobacter* in N_2-free Burk's medium with different concentrations of chemical bactericide (CB).

Chemical Bacteriocide (CB)	Different concentrations of chemical bactericide (CB) used				
	0.10%	0.20%	0.30%	0.40%	0.50%
CB*	+ + +	+ + +	+ + +	+	-

CB*:-Bactinash-200, +++ (Very good), ++ (Good), + (Seen), - (Not seen)

Table 8: Growth of *Azotobacter* in N_2-free Burk's medium with different concentrations of bio-pesticides (BP).

Bio Pesticide (BP)	Different concentrations of bio-pesticides (BP) used					
	0.3%	0.6%	0.9%	1.2%	1.5%	1.8%
BP*	+ + +	+ + +	+ + +	+ +	+	-

BP*:-Neem seed oil;   +++ (Very good), ++ (Good), + (Seen), - (Not seen)

Table 9: Morphologically study of the potent strain.

Morphological study	Strain activities
Colony morphology	Rod shaped
Consistency	Gummy
Size	Approx 2.01 mm
Pigment	Creamy white
Mortality	Non-motile
Encystment	Seen
Spore forming	Yes
PHB (Poly hydroxyl butarate)	Positive
Gram staining	Negative
Capsule	Positive
Aerobic	Yes

Table 10: Bio-chemicals study of the potent strain in different supplemented medium.

Bio-chemical study	Strain Activities
Manitol	+
Rhamnose	-
Starch hydrolysis	+
Catalase test	+
Polysaccharide	Yes
Gelatin test	-
Amylase test	+
Hydrogen sulphaide (H_2S) test	In presence of cysteine (0.1%)-Positive
Indole test	-
Methyl red test	-
VP (Voges Proskauer) test	-
Curdling in litmus milk	+
Cellulose	-
Pectinase	-
Nitrate to nitrite test	+
De-nitrification test	-
Arabinose	-
Pentose	-
Citrate malate	-
Succinate	-
Keto-glutarate	-

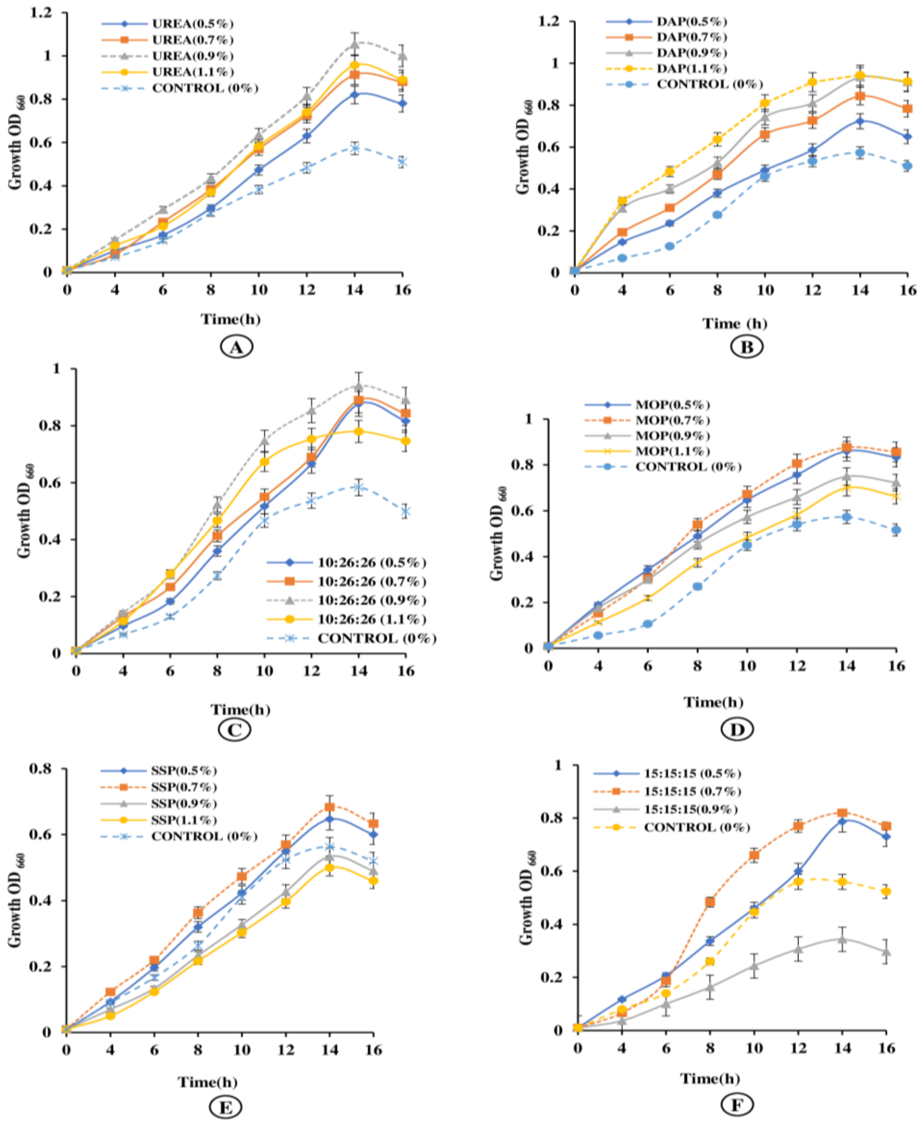

Fig. 1. Growth of *Azotobacter* in N2-free Burk's medium with different concentrations of chemical fertilizers (CFs) A. UREA, B. DAP, C. 10:26:26 (NPK), D. MOP, E. SSP, F. 15:15:15 (NPK). Data represent Mean ± S.D.

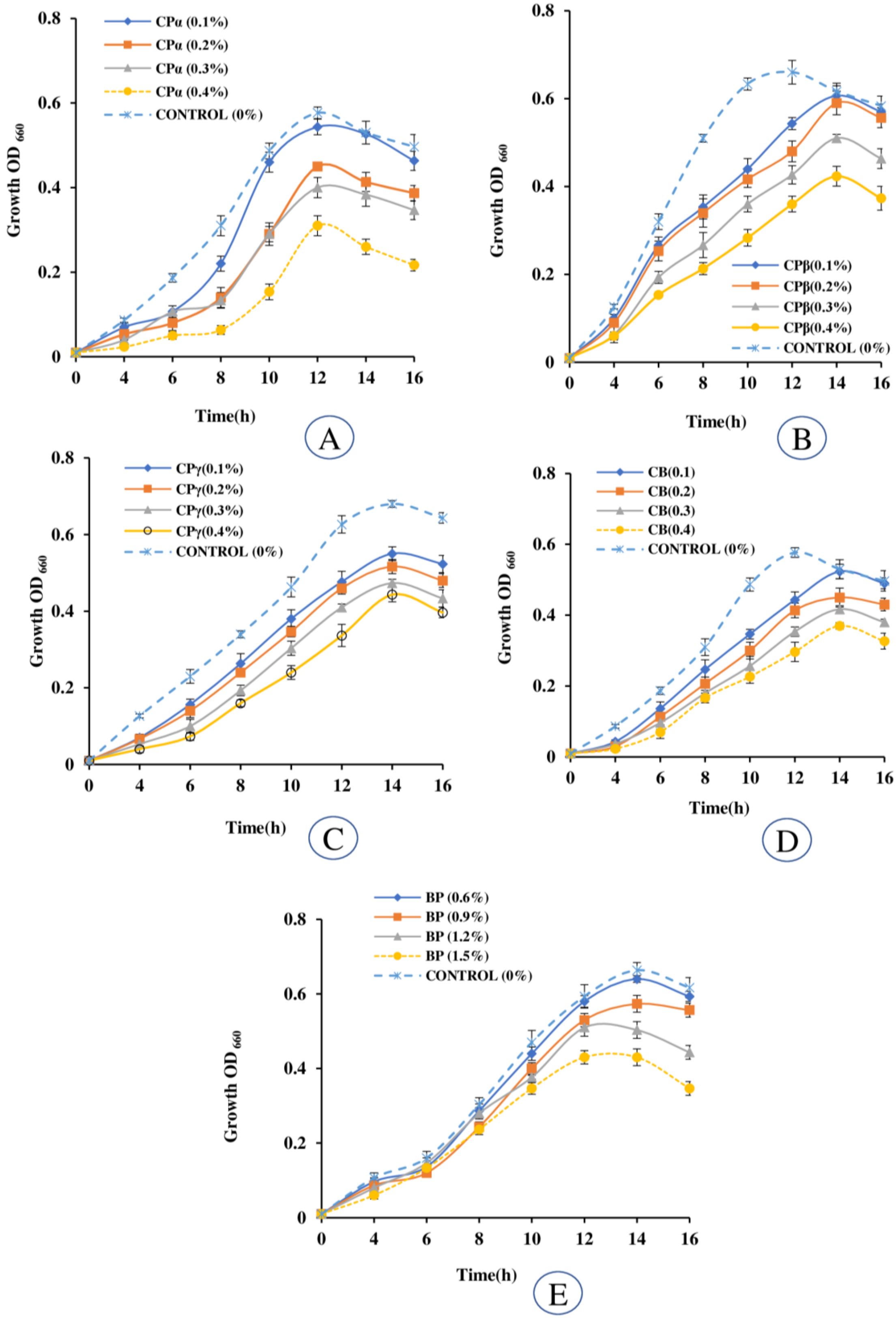

Fig. 2. Growth of *Azotobacter* in N2-free Burk's medium with different concentrations of chemical pesticides (CPs), chemical bactericide (CB) and bio-pesticide (BP) A. CP α- Tiger 500, B. CP β- Fighter 10, C. CP γ-Superkillar 10, D. CB-Bacterinash 200, E. BP-Neem Seed Oil. Data represent Mean ± S.D.

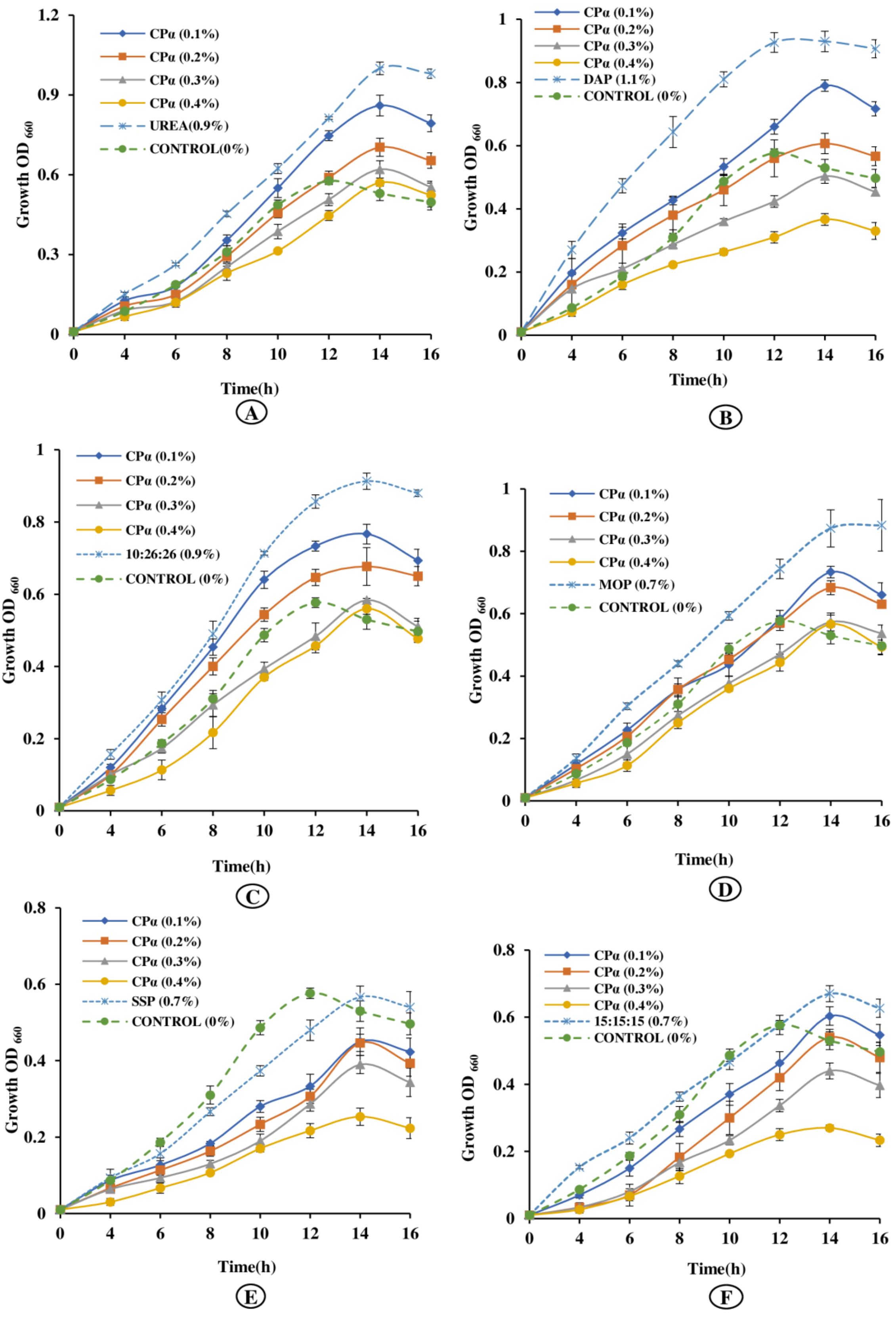

Fig. 3. Growth of *Azotobacter* in N2-free Burk's medium with optimum concentration of chemical fertilizers (CFs) and different concentrations of CP α- Tiger 500 A. UREA (0.9%), B. 10:26:26 (0.9%), C. MOP (0.7%), D. DAP (1.1%), E. 15:15:15 (0.7%) F. SSP (0.7%). Data represent Mean ± S.D.

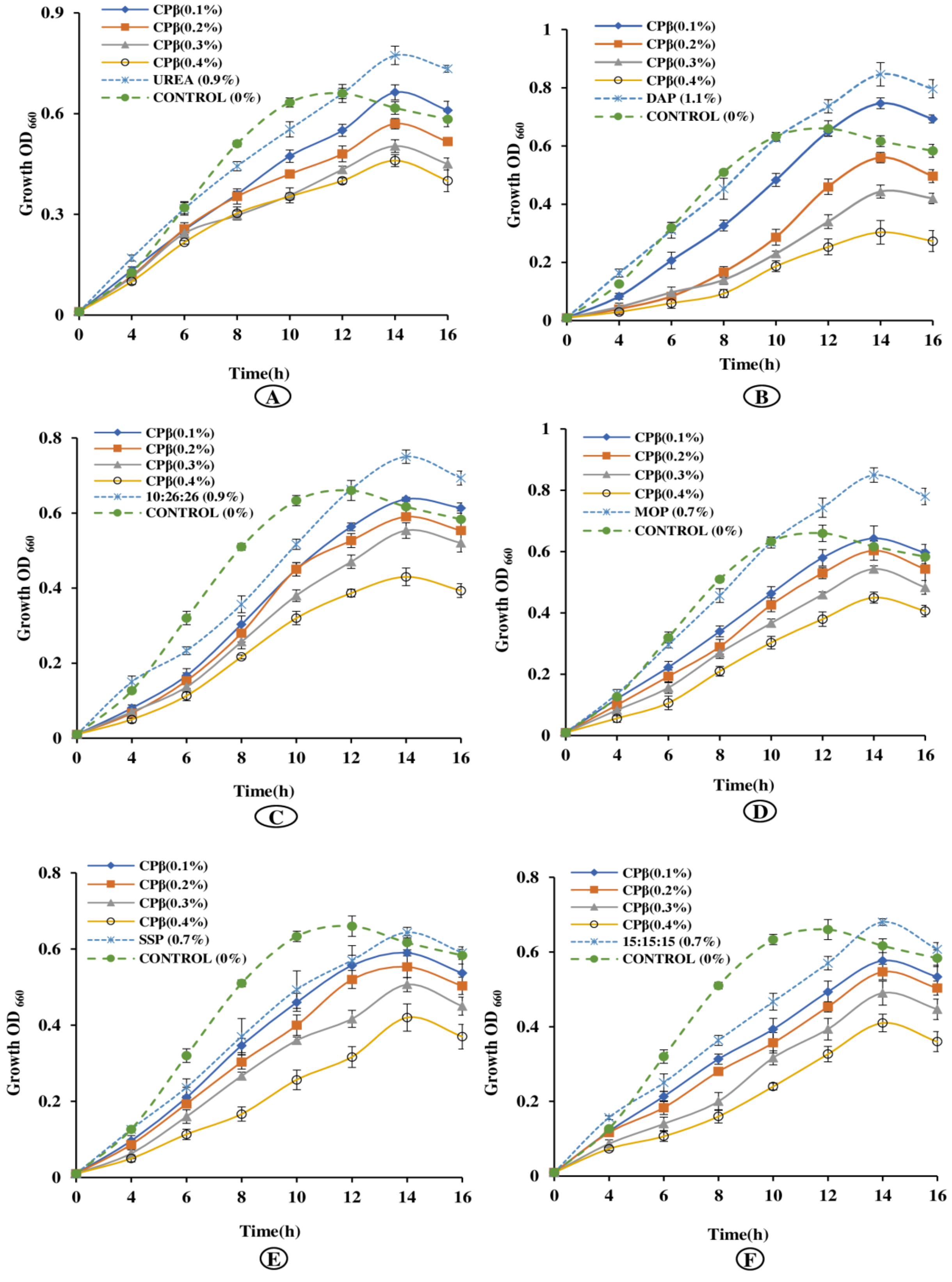

Fig. 4. Growth of *Azotobacter* in N2-free Burk's medium with optimum concentration of chemical fertilizers (CFs) and different concentration of CP β- Fighter 10 A. UREA (0.9%), B.DAP (1.1%), C. 10:26:26 (0.9%), D. MOP (0.7%), E. SSP (0.7%), F. 15:15:15 (0.7%). Data represent Mean ± S.D.

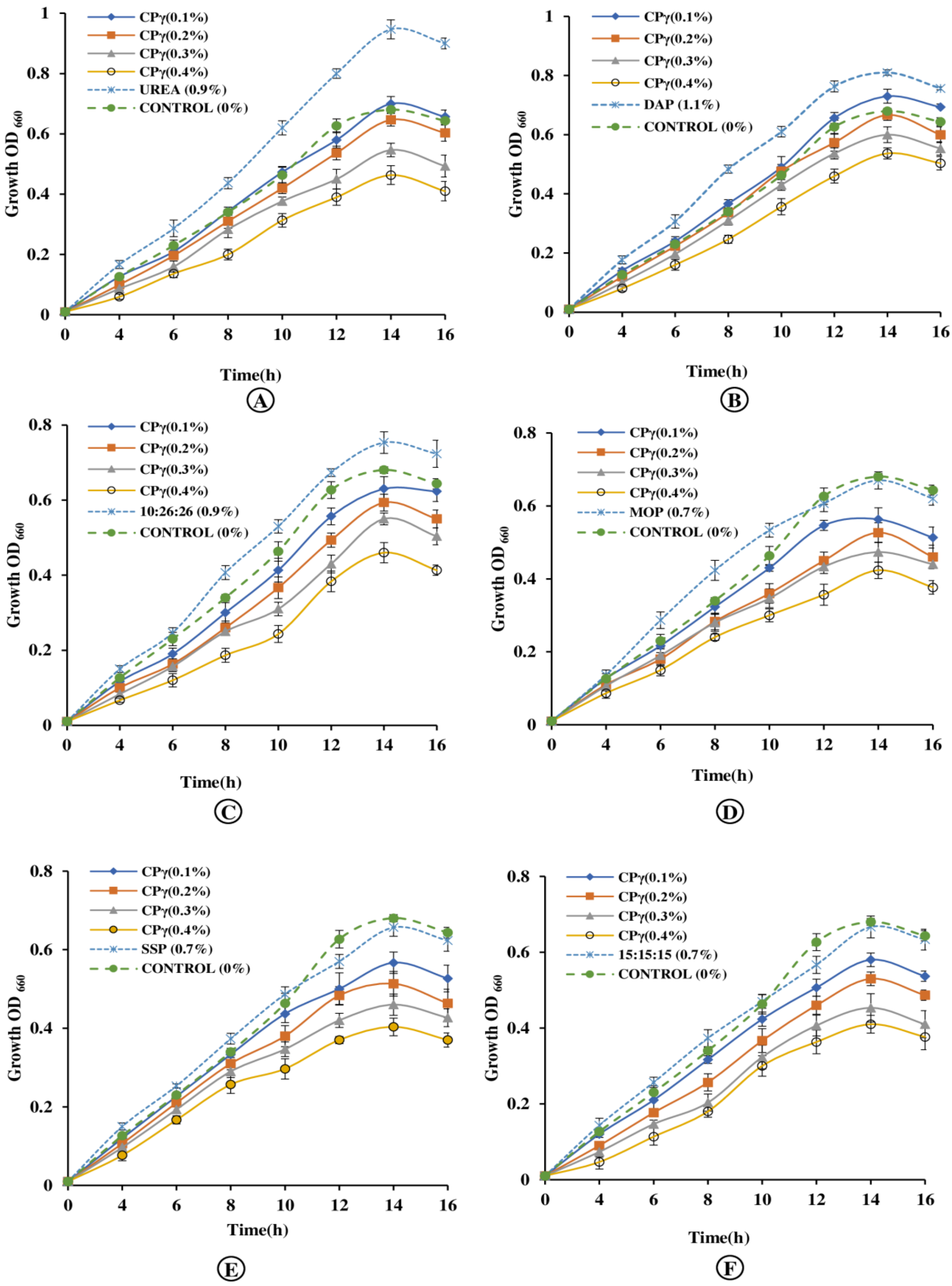

Fig. 5. Growth of *Azotobacter* in N2-free Burk's medium with optimum concentration of chemical fertilizers (CFs) and different concentrations of CP γ-Superkillar 10 A. UREA (0.9%), B. DAP (1.1%), C. 10:26:26 (0.9%), D. MOP (0.7%), E. SSP (0.7%), F. 15:15:15 (0.7%). Data represent Mean ± S.D.

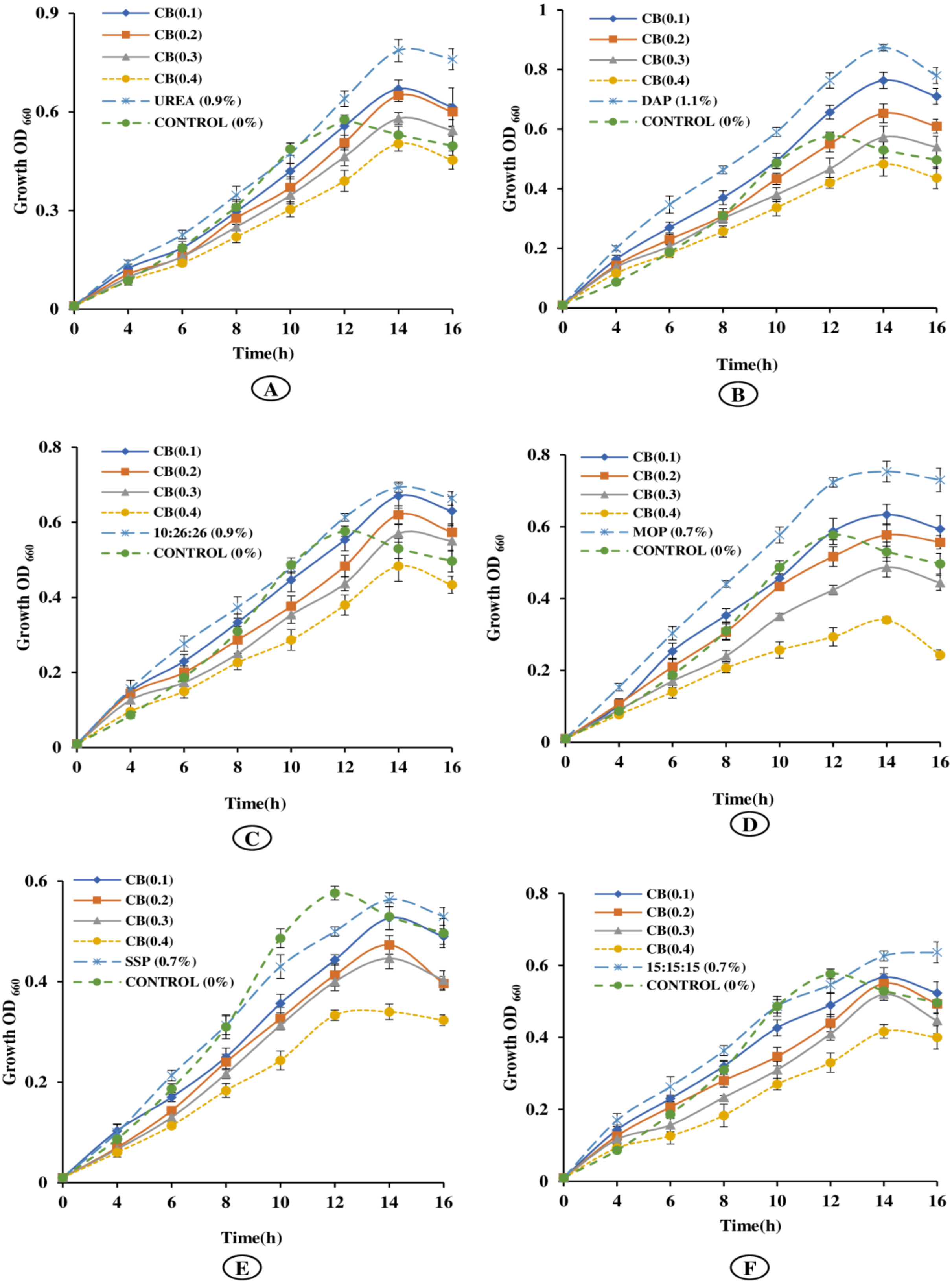

Fig. 6. Growth of *Azotobacter* in N2-free Burk's medium with optimum concentration of chemical fertilizers (CFs) and different concentrations of CB-Bacterinash 200 A. UREA (0.9%), B. DAP (1.1%), C. 10:26:26 (0.9%), D. MOP (0.7%), E. SSP (0.7%), F. 15:15:15 (0.7%). Data represent Mean ± S.D.

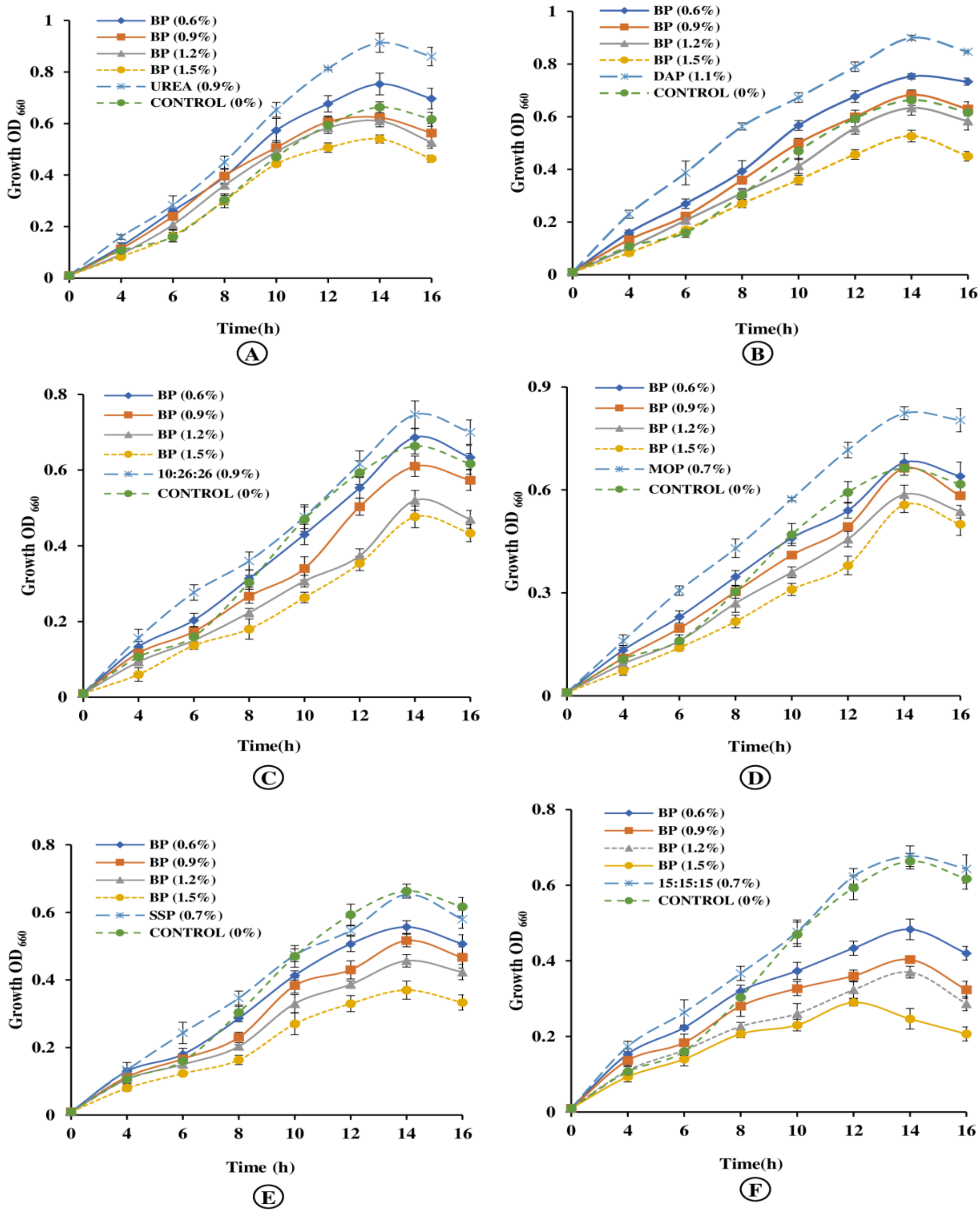

Fig. 7. Growth of *Azotobacter* in N2-free Burk's medium with optimum concentration of chemical fertilizers (CFs) and different concentrations of BP-Neem Seed Oil A. UREA (0.9%), B. 10:26:26 (0.9%), C. MOP (0.7%), D. DAP (1.1%), E. SSP (0.7%), F. 15:15:15 (0.7%). Data represent Mean ± S.D.

CONCLUSIONS

Farmers have been using huge amounts of chemical fertilizers, pesticides and bactericides in vegetable fields for more yields. It disrupts the chemical balance of natural soil. Thus, a reduction of the use of aforesaid chemicals is urgently needed. The present study specifies the use of *Azotobacter* along with a minimum amount of chemical fertilizers, chemical pesticides, and chemical bactericides in the field (vegetable) for crop production. Neem seed oil would be a good alternative to chemical pesticides and bactericides. *Azotobacter* could tolerate up to 0.4% of chemical pesticides and bactericides, 0.9% UREA, 1.1% DAP, 0.9% NPK (10:26:26) and 0.7% of MOP, NPK (15:15:15), SSP and 1.5% of bio-pesticide. The *Azotobacter* could be used as a biofertilizer for better crop production and minimum environmental disturbance.

FUTURE SCOPE

The results of this investigation have an immense impact on agriculture for crop improvement.

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Conflict of Interest. The authors declare that there is no conflict of interest.

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