IJST EXPLORE International Journal of Science and Technology
CENTRE OF PROFESSIONAL RESEARCH PUBLICATIONS
ISSN 2049-7318
International Journal of Science and Technology Volume 5 No. 1, January, 2016

# Resistance against Glyphosate and 2-4-D in the Indigenous Strain of *A. Tumefaciens* "SDB0012" And Its' Association with Oxidase Inhibition

**[1]Mohamed T. Yousif, [2]Salah A. Mustafa, [3]Aziza M. Adam and [4]Yousif Assad**
[1]National Institute for Promotion of Horticultural Exports University of Gezira, Medani, Sudan P.O. Box: 20,
[2]Faculty of Agriculture-AbuNeama, University of Sinnar, Sudan.
[3]Biotechnology and Bioscience Center, Faculty of Engineering and Technology, University of Gezira.
[4]Faculty of Agricultural Sciences, University of Gezira

## ABSTRACT

This study was conducted at University of Gezira, Sudan; The National Institute for Promotion of Horticultural Exports and Faculty of Engineering and Technology in 2008-09. It aimed at examining resistance of the indigenous strain of *A. tumefaciens* "SDB0012" to application of herbicides and role of oxidase in such resistance. Pendimethalin, Glyphosate and 2-4-D were used at dilution rates of 1:200 and 1:1 of herbicide: distilled water. Only Glyphosate and 2-4-D were further investigated at dilution rates of 2:1 and 3:1. Cyanide was used as an oxidase inhibitor at dilution rates of 0.02, 0.2, 0.5 and 2.0 mg/l. It was used to inhibit oxidase activity in the bacterial suspension and on potato discs treated with the bacterium only and with herbicides. Results concluded that the bacterium "SDB0012" was sensitive to Pendimethalin at low concentrations of 1:200 and 1:1. Further investigations concluded that the bacterium resisted Glyphosate better than 2-4-D at dilution rates of 2:1 and 3:1 and that resistance to both herbicides was highly stable. Results also indicated that resistance to Glyphosate and 2-4-D was not associated and independent. Resistance to both herbicides was found positively affected by time of incubation. Results also showed that the intensity of purple color, as an indicator for oxidase, reduced as the concentration of cyanide increased from 0.02 to 2.0 mg/l. This result indicated that oxidase had no effect on resistance of the bacterium against Glyphosate and 2-4-D. Therefore, it was suggested that this resistance might be due to effects of other independent gene(s).

**Keywords**: *Glyphosate Herbicide Resistance Agrobacterium Oxidase Gene Pentrance*

## 1. INTRODUCTION

*Agrobacterium tumefaciens* is a ubiquitous soil borne pathogen responsible for the crown gall disease. It affects many species of higher plant causing severe crop losses. Plant transformation has been successful in many species such as rice, corn, cotton and citruses **[1]**. *Agrobacterium*-mediated transformation has been the best method available for DNA transfer to tissue explants. According to ISAAA, GM crops occupied 134 million hectares out of the total area of agricultural land of over 4.9 billion hectares, in 2009. Recently, an indigenous strain of *A. tumefaciens* 'SDB0012' was identified at University of Gezira, Sudan. This strain was found to express enzymes such as catalase, oxidase and urease in the bacterial suspension **[2]**. Oxidase is an enzyme that catalyzes an oxidation reduction reaction involving molecular oxygen ($O_2$) as the electron acceptor. In microbiology, it determines whether a given bacterium produces cytochrome *c* oxidases and therefore utilizes oxygen with an electron transfer chain **[3]**. It has an herbicide resistance nature, whether it is obtained from bacteria or plant and used as a selectable marker gene in different crops for *Agrobacterium tumefaciens* mediated transformation **[4]**. Therefore, resistant to herbicide in the bacterium could either be due to expression of oxidase or due to existence of resistance genes unassociated with oxidase expression **[5]**. Herbicides are an integral part of modern day agriculture as they facilitate efficient crop management.

Most of the herbicides target specific enzymes involved in metabolic pathways that are vital for plant growth and survival (Carlisle and Trevors, 1988). The enzyme 5-enolpyruvylshikimic acid-3-phosphate EPSPS confers Glyphosate resistance; it is commonly identified in *Agrobacterium* **[6]**.

This study aimed at examining resistance of the indigenous strain of *A. tumefaciens* "SDB0012" against herbicides and role of oxidase inhibition on such resistance, through:

1. To investigate response of the bacterial strain to application of herbicides such as glyphosate, 2-4, D and Pendimethalin.

2. To test, qualitatively, expression of oxidase by this strain and its role on the bacterium resistance against herbicides.

3. To examine effect of cyanide, used as oxidase inhibitor, on virulence of the bacterium using potato disc bioassay.

## 2. MATERIALS AND METHODS

This study was carried out at National Institute for Promotion of Horticultural Exports and Faculty of Engineering and Technology, University of Gezira, Sudan in 2008-09. Experiments were conducted in a septic condition using the

Complete Randomizing Design (CRD), with three replications.

**2.1. Material used**

1. The indigenous strain of *A. tumefaciens* "SDB0012", which was developed, since 2008, at the National Institute for Promotion of Horticultural Exports-University of Gezira, Sudan.

2. Three herbicides: Glyphosate, the active ingredient of Roundup; 2,4-D and Pendimethalin, the active ingredient of Stomp, were used at dilution rates of 1:200 , 1:1 , 1:2 , 1:3 for herbicides : distilled $H_2O$.

3. NASA media, the selective media for growth of *Agrobacterium*. It was consisted of the following: 28 gm nutrient agar, 30 gm sucrose and one gm chloramphinicol dissolved in one liter distilled water.

4. Yeast Manitol Agar (YMA) medium: It was prepared as follows: 0.5 gm Di potassium hydrogen orthophosphate, 0.5 gm yeast extract, 0.2 gm magnesium sulphate, 0.1 gm sodium chloride, 15 gm Agar and 10 gm manitot. A total of 26.3 gm dissolved in one liter distilled water.

4. Phenylene-Diamine-p-Dihydrochloride as indicator for presence of oxidase.

5. Sterilized potato discs (1.5 cm in diameter).

6. Cyanide, used as oxidase inhibitor with dilution rates of 0.02, 0.2 and 0.5, 1.0 and 2.0 mg/l.

**The potato disc bioassays was conducted in the different experiments, as follows**

Preparation of *Agrobacterium tumefaciens* was done three days before assay, as follows: The growth medium YM broth was prepared in flasks plugged with cotton, covered with aluminum foil and sterilized in an autoclave for 20 minutes. Then, the medium was allowed to cool and two loop of *Agrobacterium tumefaciens* was added to the media. The flasks were placed on a shaker at speed of 150 strokes per minute for 72 hours at $30^0$C. For preparation of potato discs; the end of potato tubers were cut away and cylinders cut by the cork borer into small discs and placed (4 discs per Petri dish) in bleach by gently pushing the discs into the agar using aseptic technique. one drop (0.05ml) of the prepared inoculums was added as a treatment on the tops of each disc and then add herbicide(either 2.4,D (1:200) or glyphosate (1:200), then added cyanide with different concentration (0.02, 0.2, 0.5, 1.0, 2.0) . Potato discs used as control were treated with sterilized distilled water.

The edge of each Petri dish was sealed with parafilm strips to prevent moisture loss during the incubation period. The Petri dishes were kept in the dark at 27ºC in a horizontal position all the time to keep the inoculum on the tops of the discs. The emerged tumors were counted after 21 days.

**Experiment one: Inhibition zone**

This test was conducted following **[7]** to test the effect of herbicides applied at different concentrations on growth of the *Agrobacterium.* One ml of the bacterial suspension was added to (NASA) medium in Petri dishes and left to cool at room temperature. Each herbicide was represented by three holes in the solid medium in a Petri dish. Five µl of the herbicide was added to each hole.

**Experiment two: Total count of the bacteria**

This test was conducted following **[8]**. One ml of the bacterial suspension was added to 9 ml distilled water to make a dilution rate of ($10^{-1}$), then from the same tube one ml of the suspension were taken and added to a new tube containing 9 ml distilled water to make a dilution rate of $10^{-2}$. Then, the dilution rates ($10^3$), ($10^{-4}$), ($10^{-5}$), ($10^{-6}$) were prepared following the same pattern. One ml of glyphosate and 2.4-d were added to separate tubes at a dilution rate of 1:200. Tubes containing distilled water were used as control. The Petri dishes were kept in the dark at 27 ºC in a horizontal position to keep the inocula on the tops of the Petri dish.

**Experiment three: Qualitative test for expression of Oxidase**

One colony from a 24 hrs old culture of the bacterium on nutrient agar medium was scraped with a wooden stick and rubbed on the Phenylene-Diamine-p-Dihydrochloride on filter paper on sterilized Petri dish. The presence of a purple color within 20 seconds was recorded as a positive test for oxidase.

**Experiment four: Effect of cyanide on virulence of *Agrobacterium***

In this experiment cyanide was used at the highest concentration of 2.0 mg/l. Number of tumors produced by *Agrobacterium* on potato discs was used as an indicator to estimate for effect of oxidase on virulence of this strain.

**Experiment five: Effect of oxidase inhibition on tumor production**

Cyanide was added to potato discs containing the bacterial suspension. It was used with different concentrations of 0.02, 0.2, 0.5, 1.0, 2.0 mg/l to monitor for the concentration of cyanide that might totally inhibit oxidase, without affecting the biological activity of the bacterium.

**Experiment six: Effect of oxidase inhibition on herbicide resistance**

To study effect of oxidase on glyphosate and 2,4.D resistance, cyanide was used at concentrations of 0.02, 0.2 and 0.5mg/l. Whereas, glyphosate and 2,4.D were used at a ratio of 1ml herbicide: 200ml distilled water. Potato disc bioassay was used in this experiment as described above. Cyanide, glyphosate and 2,4.D were added to sterilized potato discs (1.5 cm in diameter) immediately after addition of the

bacterial suspension. Four potato discs were used in each Petri dish. Number of tumors grown on potato discs was counted as an indicator for herbicide resistance. Treatments used included:

2 herbicides (glyphosate and 2-4 d) x 4 concentrations of cyanide (0.02, 0.2, 0.5 and 2.0 mg/l) + control (*Agrobacterium* only).

Another experiment was conducted using the same experimental design and the same treatment. In this experiment, cyanide was added the three concentrations of 0.2, 0.5 and 2.0 mg\l.

## 3. RESULTS AND DISCUSSION

### 3.1. Inhibition zone

Pendimethalin inhibited the bacterial growth on NASA media at zones of 2.8 cm and 2.6 cm, in radius, when applied at dilution rates of 1 :200 and 1:1 for Pendimethalin: distilled water, respectively; whereas the bacterium showed resistance against glyphosate and 2-4-D, at the same dilution rates, with no inhibition zones (Table 1). Pendimethalin is the active ingredient of the Stomb which is a dinitroanaline herbicide used for pre-emergent control of annual grasses and broadleaf weeds. It is available as emulsifiable concentrate, wettable powder or dispersible granule **[9]**. The bacterium failed to resist Glyphosate and 2-4-D at dilution rate of 2: 1 for herbicide: distilled water, exhibiting inhibition zones of 2.3cm and 4cm, respectively. The inhibition zones increased to 2.4cm for Glyphosate and 4.5 cm for 2-4-D when applied herbicides at the dilution rate of 3:1 to herbicide: distilled water. Results concluded that the bacterium "SDB0012" resisted Glyphosate better than 2-4-D at dilution rates of 2:1 and 3:1. Nevertheless, the two herbicides are sprayed in the field at dilution rate more than 1:200, which was far lower than the three other dilution rates used in this experiment indicating that the indigenous strain resisted Glyphosate and 2-4-D with high pentrance and expressivity. These results could encourage recognition of gene(s) conferring resistance to both herbicides in the chromosomal DNA of the bacterium to be used in transformation of selected plant species for herbicide resistance trait. This trend of crop enhancement is considered as an effective mean of weed control, since physical means of weed control are expensive **[10]**.

Globally, some microorganisms have a version of 5-*enol*pyruvylshikimate-3-phosphate synthase (EPSPS: EC 2.5.1.19, 3-phosphoshikimate 1-carboxyvinyltransferase; 5-*enol*pyruvylshikimate-3-phosphate synthetase; phospho*enol*pyruvate: 3-phosphoshikimate 5-*O*-(1-carboxyvinyl)-transferase) that is resistant to Glyphosate; whereas, resistance of microorganisms to 2-4-D is not commonly found. The version used in genetically modified crops was isolated from *Agrobacterium* strain CP4 (CP4 EPSPS) that was resistant to glyphosate. Resistance of plants to Glyphosate is a novel characteristic sine Glyphosate is a broad sense herbicide and that the half-life of Glyphosate ranges from several weeks to years, but averages two months **[11]**. Glyphosate resistant soybean were developed by cloning and insertion of the CP4 EPSPS gene into soybeans**[12]**.

### 3.2. Total count of the bacterium

As shown above, *Agrobacterium* was not able to resist Glyphosate and 2-4-D at the dilution rates of 2:1 and 3:1 for herbicide: distilled water. In this experiment, number of bacterial colonies grown on NASA medium was used to indicate expressivity of resistance against herbicides at successive dilutions of the bacterial suspension. Pendimethalin was excluded in this experiment due to sensitivity of this bacterium to this herbicide at low dilution rates. Resistance of the bacterium to glyphosate was significantly better than 2-4-D at all dilution rates of the bacterial suspension (Table 2). At the highest concentration of bacterial suspension, the Glyphosate containing media showed 78 bacterial colonies; whereas the 2-4-D containing medium showed 69 colonies. At the $6^{th}$ cycle of dilutions, the bacterial colonies decreased to 3.5 and 4 for Glyphosate and 2-4-D containing media, respectively. These results indicated that the bacterial strain"SDB0012" resisted both herbicides at high pentrance regardless of dilution of bacterial suspension.

### 3.3. Effect of incubation period on herbicide resistance

The bacterial growth coverage was found to be 90%, 46% and 3% for glyphosate, 2-4-D and pendimethalin, respectively, two days after incubation. For glyphosate and 2-4-D, the bacterium was found to overcome effects of herbicide by increasing time of incubation to seven and 15 days. The growth coverage of the bacteria grown on media containing Glyphosate and 2-4-D increased to 97% and 50%, respectively (Fig. 1). In contrast, drastic decrease in resistance to Pendimethalin was observed as the incubation period increased and that no growth of the bacterium was found after 15 days of incubation. These results indicated high stability of resistance of the bacterium to Glyphosate and 2-4-D. Results concluded that resistance to Glyphosate was twice better than that of 2-4-D and that resistance to both herbicides was positively affected by time of incubation.

### 3.4. Inhibition of oxidase by cyanide

It was possible to quantify the terminal oxidase(s) reaction using bacterial resting-cell suspensions in the manometric assay by calculating the conventional N,N,N',N'-tetramethyl-phenylenediamine (TMPD) oxidase value (micro liters of consumed per hour per milligram on dry weight **[13]**, such techniques are not available at our laboratories. Therefore, the technique of oxidase exhibition was used in this study.

Sulfide was a more potent inhibitor of a particulate preparation of cytochrome *c* oxidase followed by cyanide and azide. The only restriction of using sulfide is that it causes death of animals by inhibition of cytochrome *c* oxidase. Cyanide is competitively inhibiting the protein from functioning which results in chemical asphyxiation of cells **[14].** In this study, cyanide was used with different rates of 0.02, 0.2, 0.5and 2.0 mg/l. Biologically, cyanide is a potent inhibitor of cellular respiration, acting on mitochondrial cytochrome *c* oxidase and hence blocking oxidative phosphorylation. This prevents the body from oxidizing food to produce useful energy. The bacterium *A. tumefaciens* SDB0012 was found to produce cytochrome c oxidase sine

the color of the filter paper was changed to purple when treated with Phenylen-diamine. Results also showed that the intensity of purple color reduced as the concentration of cyanide increased from 0.02 to 2.0 mg/l; due to reduction in oxidase concentration in the bacterial suspension by increasing cyanide concentration. By increasing concentration of cyanide to 2.0 mg/l the color of the medium was changed to faint light purple color. This indicated the presence of little excess amount of oxidase (2.0 mg/l) which in turn indicated that this concentration was not sufficient to inhibit the whole amount of oxidase expressed by the *Agrobacterium*.

### 3.5. Effect of cyanide on pathogencity of *A. tumefaciens* "SDB0012"

Results of application of cyanide at the highest rate of 2.0 mg/l showed no significant differences ($\mathcal{P}$ = 0.25) with *Agrobactrium* alone, using t-test (Table 3). The bacterium showed normal growth at this concentration. Average number of tumors was 13.34 and 12.22 for the control and cyanide, respectively. Result showed that reduction of oxidase, due cyanide application, was not affected tumor production induced by *Agrobacterium*. Therefore, it was concluded that virulence of this strain was not affected by cyanide and level of oxidase at this concentration.

### 3.6. Effect of oxidase inhibition on herbicide resistance

Some of *Agrobacterium* strains collected worldwide possess genes conferring resistance against Glyphosate, this trait was used to transform plants to express enzymes such as 5-enolpy ruvylshikimic acid-3-phosphate EPSPS **[6]**. Among these was the indigenous strain of *A. tumefaciens* "SDB0012"which was considered as unique with respect to herbicide resistance as it was found to resist both Glyphosate and 2-4-D. Moreover, it was also found to express oxidase. Resistance to herbicide generates in *Agrobacterium* could either be due to present of oxidase or to expression of specific gene(s). Therefore, a priority was given in this study to identify gene(s) conferring herbicide resistance in this strain and to differentiate between expression of oxidase and resistance to herbicides, through application of cyanide at three dilution rates to potato discs containing both the bacterial suspension and one of the herbicides.

Table 4 shows resistance of *A. tumefaciens* SDB0012 against glyphosate and 2,4.D using cyanide at rates of 0.02, 0.2 and 0.5 mg/l. The highest number of tumours was obtained with Glyphosate using cyanide at a rate of 0.5 mg/l (172 tumors) followed by 0.2 mg/l (155 tumors) and 0.02 mg/l (126 tumors). Resistance against 2-4-D showed no pattern with concentration of cyanide. This result indicated that oxidase had no effect on of the bacterium against Glyphosate and 2-4-D. It was suggested that this resistance is due to effect of other gene(s). These results were in a line with **[15]** and **[6]** who manipulated separated genes of Glyphosate resistance elsewhere in the chromosomal DNA a part from the sequence than conferring expression of oxidase and transferred then to wheat and cotton. In contrast with **[4]** who mentioned that oxidase is known to have an herbicide resistance nature. On the other hand, resistance of the bacterium to Glyphosate and 2-4-D was not statistically correlated (low values of x-coefficient), which was suggested that resistance of the bacterium to Glyphosate and 2-4-D was due to separate genes.

Results presented in Fig. 2 for Glyphosate and Fig. 3 for 2-4-D were in a line with those presented in Table 4. Number of tumors produced by the bacterium was significantly affected by application of Glyphosate and 2-4-D. Application of cyanide was positively affected resistance of the bacterium against Glyphosate; whereas, it has no specific manner with resistance of the bacterium against 2-4-D. Results concluded that oxidase has no effect on resistance of the bacterium to Glyphosate and 2-4-D.

## 4. CONCLUSIONS AND RECOMMENDATIONS

Results concluded ability of the bacterium to produce oxidase. Cyanide could be used effectively to overcome oxidase activity without affecting the biological activities of the bacterium. Results also concluded that the bacterium had resistance against both Glyphosate and 2-4-D with high stability. Resistance against both herbicides was not affected by oxidase and could be due to separate genes. This study proposed molecular identification of resistance genes using molecular markers and DNA sequencing looking at construction of genes that could be used effectively in plants transformation against resistance to those herbicides.

## REFERENCES

Hidaka, T., Omura, M., Ukagi, M., Tomiyama, M., Kato, A., Ohshima, M. and Motoyoshi, F. 1990. *Agrobacterium*-mediated transformation and regeneration of *Citrus* spp. from suspension cells. *J. Breed,* 40: 199-207.

Yousif M. T., Al-Mustafa S. A. and Siddig, M.A.. 2011. Enzymatic activities, herbicide resistance and plasmid characteristics of the indigenous *Agrobacterium tumefaciens* "*SDB 0012*". *Arab Journal of Biotechnology* Volume 14, No. (1):89-100.

Michel, H. 1999. Cytochrome *c* Oxidase: Catalytic Cycle and Mechanisms of Proton Pumping-A. Discussion *Biochemistry*, *38*, 15129-15140.

Xianggan, L.I.; Sandy, L.V.; David, B.G. Nicholl, C.E. Zupan, C.; Muth, T. R.; Draper, O. and Zambryski, P. 2003. The transfer of DNA from *Agrobacterium tumefaciens* into plants, a feast of fundamental insights. *The Plant Journal*., (23): 11-28.

William, L.; Patzoldt, A.; Hager, J.; McCormick, S. and Patrick, J. T. 2006. A codon deletion confers resistance to herbicides inhibiting protoporphyrinogen oxidase.

Fu, Y. Z.; Yun-Feng, L.I. and Peilin, X.U. 2006. *Agrobacterium* mediated transformation of cotton (*Gossypium hirsutum* L.). *Biotechnology Letters* ( 28), p 1199-1207.

Collins, C.I.I.; Lyne, P.M. and., Grange, J.M. 1995. Collins and Lyne's Microbiological Methods, 7th edition. Butterwoth-Heinemann Ltd, Oxford, England. P: 493.

Collee, J. G.; Fraster, A. G.; Marmion, B. P. and Simmons, A. 1996. Practical Medical Microbiology. 14th edition., PP. 978. Longman, Singapore.

Kellogg, R.L, Nehring R., Grube A, Goss D.W. and Plotkin S. 2000. Environmental indicators of pesticide leaching and runoff from farm fields. United States Department of Agriculture Natural Resources Conservation Service. Retrieved on 2007-10-03.

Powless, Preston C. 2006. Evolved glyphosate resistance in plants: biochemical and genetic basis of resistance. *Weed Technology*, 20(2): 282-9.

Ross, M.A. and Lembi, C.A. 1985. Applied weed science. Burgess Publishing Co., Minneapolis, MN. 340 pp.

Heck, G.R. 2005. Development and Characterization of a CP4 *EPSPS*-Based, Glyphosate-Tolerant Corn Event. *Crop Sci.* 45: 329–339.

Peter, J. R. and Donald, N. 1976. Use of a Quantitative Oxidase Test for Characterizing. *Appl Environ Microbiol*, (5): 668–679.

Alonso, J.; Cardellach, F.; López, S.; Casademont, J. and Miró, O. 2003. "Carbon monoxide specifically inhibits cytochrome *c* oxidase of human mitochondrial respiratory chain". *Pharmacology and Toxicology p: 142-146*.

Hu, T.S., Metz, C., Chay, H.P., Zhou, N., Biest, G., Chen, M.; Cheng, X., Feng, M., Radionenko, F. and Lu, J.F. 2003. *Agrobacterium* mediated large-scale transformation of wheat (*Triticum aestivum* L.) using glyphosate selection than its glanded counterpart. *Pestic. Biochem. Physiol.*, 31: 269-275.

**Table 1. Response Of The Indigenous *Agrobacterium* To The Three Herbicides Applied At Different Dilution Rates.**

| Dilution rate (Herbicide: distilled water) | Inhibition zone (cm) | | |
|---|---|---|---|
| | Glyphosate | 2-4-D | Pendimethalin |
| 1:200 | 0 | 0 | 2.8 |
| 1:1 | 0 | 0 | 2.6 |
| 2: 1 | 2.3 | 4 | *nt** |
| 3: 1 | 2.4 | 4.5 | *nt* |

*= not tested, as the bacterium could not be able to resist the herbicide at lower concentrations.

**Table 2. Number of bacterial colonies at different dilution rates of the bacterial suspension, applied with Glyphosate and 2-4-D.**

| Herbicide | Dilution rate | | | | | |
|---|---|---|---|---|---|---|
| | $10^{-1}$ * | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ |
| Glyphosate | 78 | 53 | 30 | 15 | 7 | 3.5 |
| 2-4-D | 69 | 47 | 24 | 14 | 6 | 4 |

*Subsequent dilution of the bacterial suspension was done by removing 1/10 of the suspension to a new tube containing 9 ml of distilled water in the successive cycles.

**Table 3. Effect of cyanide on number of tumours grown on potato discs by *A. tumefaciens* "SDB0012"**

| Treatments | Average number of tumours |
|---|---|
| *Agrobacterium* alone | 13.34 |
| *Agrobacterium* + cyanide (2.0 mg/l) | 12.22 |
| $\mathcal{P}$ | 0.52* |
| S.E. | ±0.64 |

*Using t-test

**Table 4. Effect of cyanide concentrations on tumor production induced by *Agrobacterium* on potato discs**

| Treatment | Number of tumor in different Concentration of cyanide (mg/l) | | | Average number of tumors |
|---|---|---|---|---|
| | 0.02mg/l | 0.2mg/l | 0.5mg/l | |
| *Agrobactirum* + glyphosate (1:200) | 126*c* | 155*bc* | 172*a* | 151 |
| *Agrobactirum* + 2,4.D (1:200) | 166*b* | 117*cd* | 159*ab* | 147.33 |
| Overall main | 146 | 136 | 165.5 | |
| X-coefficient | 19.83 | 1.19 | -96.42 | |

S.E =± 0.79 $\mathcal{P}$=0.05 D = 13.00 LSD = 79.10 C.V % = 20.06

* Glyphosate and 2,4.D were applied at a rate of 1 herbicide: 200 distilled water.

**Petri dishes containing *Agrobacterium* alone were used as control.

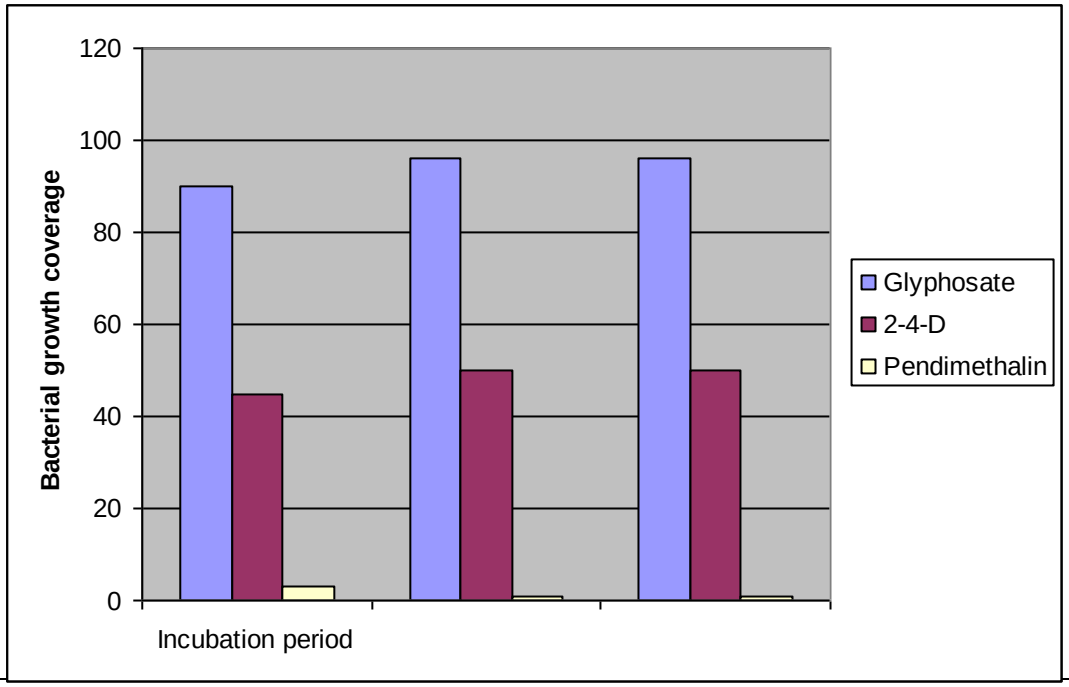
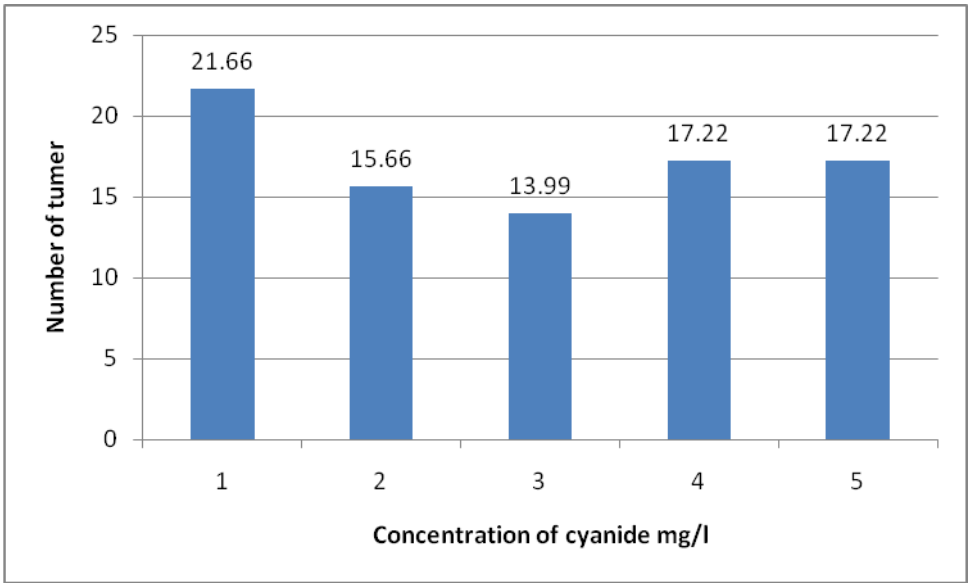


**Fig. 1. Effect of incubation period on herbicide resistance**

- Incubation periods used were 2, 7 and 15 days, from left to right.




Where:

1 = control, 2 = glyphosate alone, 3 = glyphosate + cyanide 0.02 mg/l, 4 = glyphosate + cyanide 0.2 mg/l, 5 = glyphosate + cyanide 0.5 mg/l.

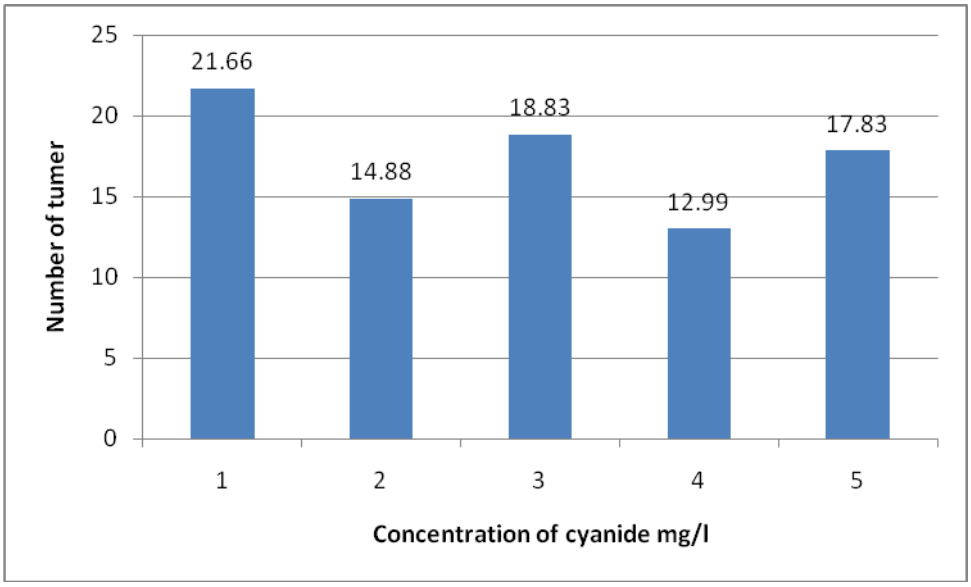



**Fig. 3. Effects of cyanide and 2-4-D on tumor production**

* Where:

1 = control, 2 = 2,4.D lone, 3= 2,4.d+cyanide 0.02 mg/l, 4 = 2,4.D +cyanide 0.2 mg/l,

5 = 2,4.D +cyanide 0.5 mg/l.