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Full Length Research Paper

## ***In vitro* regeneration of *Tripsacum laxum* Nash**

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***Tripsacum laxum* Nash (Guatemala grass) is widely used globally as a forage crop. Its strong roots and long period of growth and utilization allow it to be harvested for many years, and given its high yield, high nutritional value, and good taste. Here the peduncles of *T. laxum* Nash were used as explants to induce shoots and then efficient shoot proliferation and regeneration system were established for the first time. Multiple shoots were proliferated on Murashige and Skoog (MS) medium to establish, for the first time, an efficient shoot proliferation and plant regeneration systems. Optimal shoot proliferation medium was MS with 3.0 mg/L 6-benzyladenine (BA) and 0.2 mg/L  $\alpha$ -naphthaleneacetic acid (NAA), resulting in a shoot proliferation coefficient of 11.0 within 45 days. Optimal rooting medium was MS with 0.1 mg/L NAA and/or 0.1 mg/L indole-3-butyric acid (IBA), inducing 100% root formation from shoots within 30 days. The *in vitro* young roots, leaf sheaths and shoot bases were also used as explants, to induced embryogenic callus. The results showed that MS medium with 1.0 mg/L thidiazuron (TDZ) and 0.2 mg/L BA induced most shoots, with the least callus. Shoot bases induced beige-white callus and shoots directly on MS medium with 1.0 mg/L TDZ and 0.2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), while leaf sheaths induced beige-white callus and shoots directly on MS medium with 1.0 mg/L TDZ and 0.2 mg/L BA. The rooted plantlets showed 99.3% survival when transplanted into a substrate of vermiculite: peat soil (1:3, v/v).**

**Key words:** *Tripsacum laxum*, axillary shoots, callus, adventitious shoots, rooting, regeneration.

### INTRODUCTION

The genus *Tripsacum* (Maydeae tribe, Panicoideae, Gramineae) includes 16 species that grow in many ecologically distinct niches and habitats that are typically distributed in tropical and subtropical regions (Gray, 1974; Wet et al., 1985). Since *Tripsacum* has a common ancestor with maize and teosinte, it may be important to better understand the origin and evolution of maize. *Tripsacum* is a perennial warm-season C<sub>4</sub> type of grass

that is often used to produce high-quality forage and biomass energy, and control soil erosion (Zhao et al., 2020). *Tripsacum laxum* Nash (Guatemala grass) is widely used globally as a forage crop (Munyasi et al., 2015; Maleko et al., 2018; Klapwijk et al., 2020). Its strong roots and long period of growth and utilization allow it to be harvested for many years, and given its high yield, high nutritional value, and good taste, it is suitable

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for cutting green feed or process into silage material, and can thus be used as feed for cattle, ducks, geese and pigs in the limestone soils and a seasonally dry habitat (Wilkes, 1972; Boonman, 1993; Boschini-Figueroa and Vargas-Rodríguez, 2018). The roots of *T. laxum* develop well, and when tilled into soil and used as organic matter, this improves the physical and chemical structure of the soil, so it is often used as a multi-year cover crop (Shem et al., 1995). After *T. laxum* was introduced to China, it is now a major source of forage feed (Jiang et al., 2002; Zhong et al., 2011). Recently, it has been completed in chloroplast genome and evolutionary relationship analysis showed that it is more closely related to *Tripsacum dactyloides* (Luo et al., 2021).

The chromosome number of *T. laxum* is  $2n = 72$  (Dodds and Simmonds, 1946; Zhong et al., 2011). Although most chromosomes are bivalents, there are multiple chromosomal irregularities, ultimately resulting in male sterility (Dodds and Simmonds, 1946). *T. laxum* is rarely propagated by stem cuttings or rhizomes (Guyadeen, 1951; Wilkes, 1972). However, the stems tend to shrink and are prone to bacterial infections (Tuley, 1961; Schieber, 1975; Asudi et al., 2015). To resolve limitations associated with proliferation and to overcome disease-related problems, the establishment of an *in vitro* regeneration system would allow this plant to be mass propagated and to create a platform that would allow for its genetic improvement through transgenic strategies. To our knowledge, there are no studies on the tissue culture or related biotechnologies of *T. laxum*. In this study, for the first time, the young peduncles of *T. laxum* was employed as explants to induce shoots that were then proliferated to establish an efficient *in vitro* regeneration system.

## MATERIALS AND METHODS

### Establishment of *in vitro* tissue culture

*T. laxum* plants growing on a farm in Guigang city, Guangxi province were brought back to Guangzhou in 2010. All the studies comply with relevant institutional, national, and international guidelines and legislation. It has been specified under the appropriate permissions and licenses for the collection of plant specimens. Plants were propagated by cutting and grown in a test field of South China Botanical Garden, Guangzhou, Guangdong Province. The plants flowered every year but no seed were produced (Figure 1a). Stems were cut into 30 cm long cuttings, planted in a field and allowed to grow naturally. Plants were identified by Dr. Qing Liu, a botanist in South China Botanical Garden. When the plants began to flower, between March and April of 2016, young inflorescences of *T. laxum* were removed with a surgical knife (Figure 1b). The peduncles segments (5 cm long) were the first surface disinfected with 75% ethanol using cotton balls, dipped into 0.1% (w/v) mercuric chloride solution ( $\text{HgCl}_2$ ) for 10 min, then washed three times with sterile distilled water. Surface-disinfected explants (2-3 cm long peduncles) were inoculated into Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) containing 1.0 mg/L 6-benzyladenine (BA) and 30 g/L sucrose. Medium pH was adjusted to 6.0 before being solidified

with 0.7% (w/v) agar (Sigma-Aldrich, St. Louis, MO, USA), then autoclaved at 121°C for 20 min. Culture jars (height = 10 cm; diameter = 8 cm) were placed in an air-conditioned culture room at  $25 \pm 2^\circ\text{C}$  with a 12-h photoperiod and  $100 \mu\text{M m}^{-2} \text{s}^{-1}$  fluorescent light (Philips, Tianjin, China). Tissue culture conditions were identical to those used for another grass *Lepturus repens* (Xiong et al., 2021). After 15 days in culture, some axillary shoots buds (Figure 1c) were induced from peduncle internodes. Axillary shoots were subcultured on the same medium every 45 days. When sufficient stock was proliferated, experiments were initiated.

### Effects of plant growth regulators on axillary shoot proliferation

Using a similar technique as was employed for *Scaevola sericea* (Liang et al., 2020), axillary shoot clusters were cut into smaller clusters, each with three shoots. These were “inoculated onto MS medium containing different combinations and concentrations of plant growth regulators (PGRs) for axillary shoot proliferation (Table 1). For each treatment, 10 jars were used. Each jar contained three shoot clusters. After culture for 45 days, axillary shoot proliferation coefficient (SPC) was assessed as: number of axillary shoots after proliferation for 45 days/number of axillary shoots before proliferation” (Liang et al., 2020).

### Adventitious root formation

Adventitious root formation used a similar method as was employed for *S. sericea* (Liang et al., 2020). Axillary shoots were separated and cultured on rooting medium ( $\frac{1}{2}\text{MS}$ ) “supplemented with different concentrations and combinations of indole-3-butyric acid (IBA) and  $\alpha$ -naphthaleneacetic acid (NAA) (Table 2). In each treatment, 10 jars were inoculated and each jar contained three shoots. PGR-free  $\frac{1}{2}\text{MS}$  medium was used as the control. After 15 and 30 days of culture, rooting percentage was observed and assessed, as follows (Liang et al., 2020):

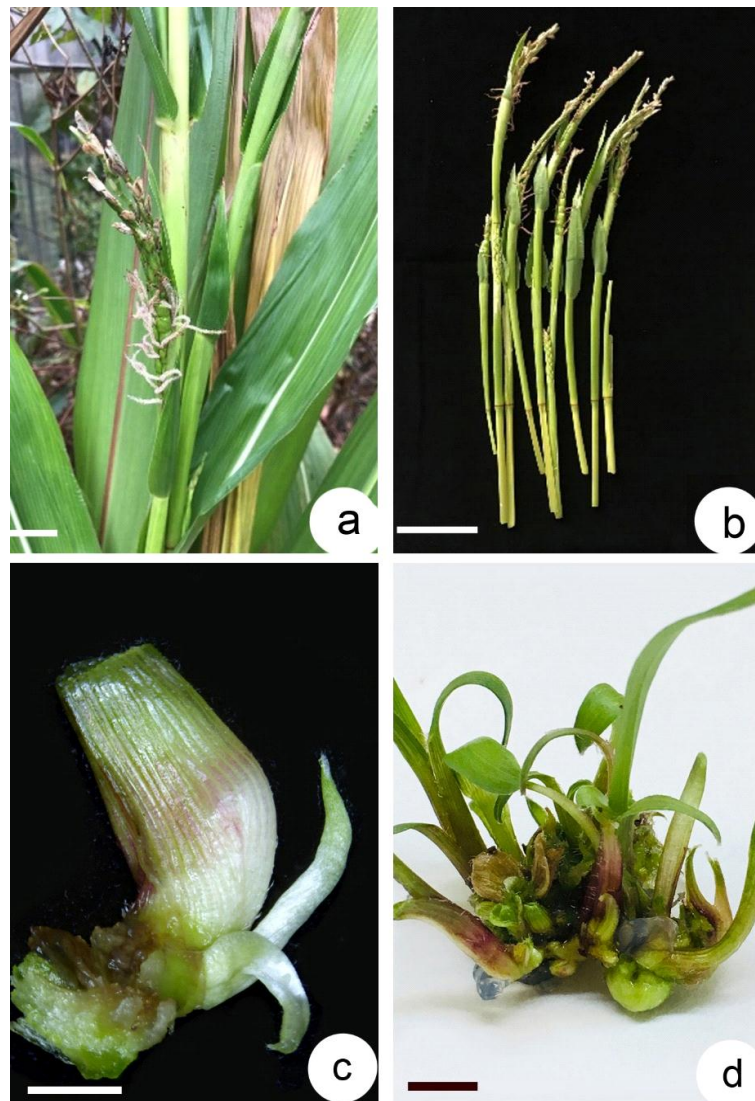
(number of buds that rooted after 30 days/number of inoculated buds)  $\times$  100%.

### Effects of plant growth regulators on callus induction from three explant types

Young roots, young leaf sheaths and shoot bases were used as explants. Roots were derived from 15 day-old plantlets that had been rooted in  $\frac{1}{2}\text{MS}$  medium with 0.1 mg/L NAA. Roots were cut into 1.0 cm long explants. The young leaf sheaths and shoot bases were derived from shoots that had been proliferated on MS medium with 1.0 mg/L BA for 45 days. These tissues were cut into explants  $0.5 \text{ cm}^2$  in size and inoculated onto MS-based media with different plant growth regulators (PGRs) to induce callus and observe differentiation after 30 days (Tables 3 to 5).

### Acclimatization and transplantation

An acclimatization protocol, similar to that which was used for *S. sericea* (Liang et al., 2020), was employed. Culture jars with shoots that were rooted in  $\frac{1}{2}\text{MS}$  medium with 1.0 mg/L IBA for 30 days were transferred to natural light for 7 days. Using tap water, agar was gently rinsed off roots. Rooted plantlets were transplanted into plastic pots (height and diameter = 10 cm) containing yellow mud and peat soil (1:1, v/v), or peat and vermiculite (3:1, v/v). A single plantlet was planted in each plastic pot, and each treatment had 30 plantlets. Plants were watered every morning with tap water. After



**Figure 1.** Induction of axillary shoots from *Tripsacum laxum* peduncle explants derived from immature inflorescences. (a) Flowering plants growing outside a greenhouse (March, 2019); (b) peduncle explants collected from immature inflorescences of plants growing outdoors; (c) a few axillary shoot buds were induced from a peduncle explant on MS medium with 1.0 mg/L BA within 30 days; (d) multiple axillary shoots were induced from a peduncle explant on MS medium with 3.0 mg/L BA and 0.1 mg/L NAA within 60 days. Bars: 2 mm (c, d); 1 cm (a, b).

Source: Authors

30 days, plantlet height was determined. Survival percentage of transplanted plantlets was assessed as (Liang et al., 2020):

$(\text{number of living plantlets before transplanting} / \text{number of living plantlets after transplanting for 30 d}) \times 100\%$ .

#### Statistical analyses

All experiments were repeated three times within one week. Data are reported as mean  $\pm$  standard deviation (SD). Means were statistically analyzed by one-way analysis of variance (ANOVA).

Treatment means were considered to be significantly different from controls after applying Duncan's multiple range test ( $P \leq 0.05$ ) using SPSS v. 19.0 (IBM, New York, NY, USA).

## RESULTS

### Shoot proliferation on different media

BA induced shoots more effectively than KIN, as assessed by SPC, but not when its concentration

**Table 1.** Effect of PGRs in MS medium on SPC of *Tripsacum laxum* after 45 days.

PGRs (mg/L)	SPC
BA 1.0	4.9 ± 0.4 <sup>d</sup>
BA 3.0	6.9 ± 0.4 <sup>b</sup>
BA 5.0	7.0 ± 0.4 <sup>b</sup>
BA 1.0 + NAA 0.1	6.1 ± 0.3 <sup>c</sup>
BA 3.0 + NAA 0.1	11.0 ± 0.5 <sup>a</sup>
BA 5.0 + NAA 0.1	10.8 ± 0.5 <sup>a</sup>
KIN 1.0	3.1 ± 0.4 <sup>f</sup>
KIN 3.0	5.1 ± 0.5 <sup>d</sup>
KIN 5.0	5.2 ± 0.3 <sup>d</sup>
KIN 1.0 + NAA 0.1	4.0 ± 0.3 <sup>e</sup>
KIN 3.0 + NAA 0.1	5.9 ± 0.4 <sup>c</sup>
KIN 5.0 + NAA 0.1	6.2 ± 0.3 <sup>c</sup>

Values represent means ± SD. Different letters within a column indicate significant differences according to the Duncan's multiple range test ( $P \leq 0.05$ ). n = 30 per treatment. BA, 6-benzyladenine; KIN, kinetin; NAA,  $\alpha$ -naphthaleneacetic acid; SPC, shoot proliferation coefficient.

Source: Authors

**Table 2.** Rooting of *Tripsacum laxum* in 1/2MS medium supplemented with different auxins.

Auxins (mg/L)	Rooting percentage at different culture periods (d)	
	15 days	30 days
Control	0 <sup>c</sup>	78.7 ± 7.3 <sup>b</sup>
NAA 0.2	67.4 ± 5.3 <sup>b</sup>	100 <sup>a</sup>
IBA 0.2	74.3 ± 6.7 <sup>b</sup>	100 <sup>a</sup>
NAA 0.2 + IBA 0.2	100 <sup>a</sup>	100 <sup>a</sup>

Values represent means ± SD. Different letters within a column indicate significant differences according to Duncan's multiple range test ( $P \leq 0.05$ ). n = 30 per treatment. IBA, indole-3-butyric acid; NAA,  $\alpha$ -naphthaleneacetic acid.

Source: Authors

**Table 3.** Effect of PGRs in MS medium on callus induction and adventitious bud differentiation from young root explants of *Tripsacum laxum* after culture for 30 days.

PGRs (mg/L)	Roots forming callus (%)	Callus differentiation into shoots (%)
2,4-D 1.0	2.8 ± 1.2 <sup>d</sup>	0 <sup>c</sup>
NAA 1.0	2.4 ± 1.3 <sup>d</sup>	0 <sup>c</sup>
BA 1.0	3.6 ± 1.2 <sup>d</sup>	0 <sup>c</sup>
BA 1.0 + NAA 0.2	4.1 ± 1.4 <sup>d</sup>	0 <sup>c</sup>
TDZ 1.0	10.3 ± 1.1 <sup>c</sup>	1.6 ± 0.5 <sup>b</sup>
TDZ 1.0 + NAA 0.2	13.3 ± 0.9 <sup>b</sup>	2.2 ± 0.6 <sup>b</sup>
TDZ 1.0 + BA 0.2	20.0 ± 1.2 <sup>a</sup>	3.8 ± 0.8 <sup>a</sup>

Values represent means ± SD. Different letters within a column indicate significant differences according to Duncan's multiple range test ( $P \leq 0.05$ ). n = 30 per treatment. 2,4-D, 2,4-dichlorophenoxyacetic acid; BA, 6-benzyladenine; NAA,  $\alpha$ -naphthaleneacetic acid; PGR, plant growth regulator; TDZ, thidiazuron.

Source: Authors

exceeded 3.0 mg/L (Table 1). When 6-benzyladenine (BA) was supplemented with 0.2 mg/L NAA, axillary shoot

**Table 4.** Effect of PGRs in MS medium on callus induction and adventitious shoot formation from young leaf sheath explants of *Tripsacum laxum* after culture for 30 days.

PGRs (mg/L)	Callus induction (% of explants)	Number of shoots/explant (%)	Callus induction and differentiation
2,4-D 1.0	3.5 ± 1.2 <sup>d</sup>	0 <sup>c</sup>	Little callus, no shoots
NAA 1.0	3.3 ± 1.1 <sup>d</sup>	0 <sup>c</sup>	Little callus, no shoots
BA 1.0	4.3 ± 1.3 <sup>d</sup>	0 <sup>c</sup>	Little callus, no shoots
TDZ 1.0	3.5 ± 1.4 <sup>d</sup>	0 <sup>c</sup>	Little callus, no shoots
BA 1.0 + NAA 0.2	4.4 ± 1.5 <sup>d</sup>	0 <sup>c</sup>	Little callus, no shoots
BA 2.0 + NAA 0.2	5.6 ± 1.6 <sup>d</sup>	0 <sup>c</sup>	Little callus, pink, no shoots
TDZ 0.2 + BA 1.0	15.1 ± 1.4 <sup>c</sup>	2.3 ± 0.5 <sup>b</sup>	Beige-white, shoots
TDZ 0.2 + BA 2.0	16.7 ± 1.6 <sup>c</sup>	2.1 ± 0.6 <sup>b</sup>	Beige-white, shoots
TDZ 1.0 + NAA 0.2	4.6 ± 1.3 <sup>d</sup>	0 <sup>c</sup>	Little callus, no shoots
TDZ 2.0 + NAA 0.2	6.3 ± 1.5 <sup>d</sup>	0 <sup>c</sup>	Little callus, no shoots
TDZ 1.0 + BA 0.2	30.8 ± 3.5 <sup>a</sup>	4.6 ± 0.8 <sup>a</sup>	Beige white, shoots
TDZ 2.0 + BA 0.2	23.1 ± 2.4 <sup>b</sup>	3.5 ± 0.7 <sup>a</sup>	Beige white, shoots

Values represent means ± SD. Different letters within a column indicate significant differences according to Duncan's multiple range test ( $P \leq 0.05$ ). n = 30 per treatment. 2,4-D, 2,4-dichlorophenoxyacetic acid; BA, 6-benzyladenine; NAA,  $\alpha$ -naphthaleneacetic acid; PGR, plant growth regulator; TDZ, thidiazuron.

Source: Authors

**Table 5.** Effect of PGRs in MS medium on callus induction and differentiation into shoot buds from shoot bases of *Tripsacum laxum* after culture for 30 days.

PGRs (mg/L)	Callus induction (% of explants)	Callus description	Number of adventitious shoots/explant
2,4-D 1.0	91.7 ± 5.2 <sup>a</sup>	Brown, hyperhydric	0 <sup>f</sup>
TDZ 1.0	41.7 ± 2.6 <sup>e</sup>	Yellow, compact	0 <sup>f</sup>
BA 1.0	11.3 ± 1.5 <sup>f</sup>	Compact	0 <sup>f</sup>
2,4-D 1.0 + BA 0.2	75.1 ± 3.3 <sup>c</sup>	Brown	1.1 ± 0.3 <sup>e</sup>
2,4-D 1.0 + NAA 0.2	92.7 ± 7.3 <sup>a</sup>	Friable, pink	1.3 ± 0.3 <sup>e</sup>
2,4-D 1.0 + TDZ 0.2	91.5 ± 6.2 <sup>a</sup>	Beige-white, yellow	2.2 ± 0.4 <sup>d</sup>
TDZ 1.0 + BA 0.2	50.0 ± 3.4 <sup>d</sup>	Beige, yellow	5.3 ± 0.4 <sup>b</sup>
TDZ 1.0 + NAA 0.2	75.0 ± 3.2 <sup>c</sup>	Beige-white	3.7 ± 0.5 <sup>c</sup>
TDZ 1.0 + 2,4-D 0.2	83.3 ± 3.5 <sup>b</sup>	Beige-white, yellow	9.2 ± 0.3 <sup>a</sup>

Values represent means ± SD. Different letters within a column indicate significant differences according to Duncan's multiple range test ( $P \leq 0.05$ ). n = 30 per treatment. 2,4-D, 2,4-dichlorophenoxyacetic acid; BA, 6-benzyladenine; NAA,  $\alpha$ -naphthaleneacetic acid; PGR, plant growth regulator; TDZ, thidiazuron.

Source: Authors

number increased significantly (Table 1 and Figure 1d), with 3.0 mg/L BA and 0.2 mg/L NAA assessed as the optimal medium for shoot proliferation (Figure 3a). When culture period was extended to 45 day, some shoots formed roots at their base (Figure 3b), suggesting that rooting was easy.

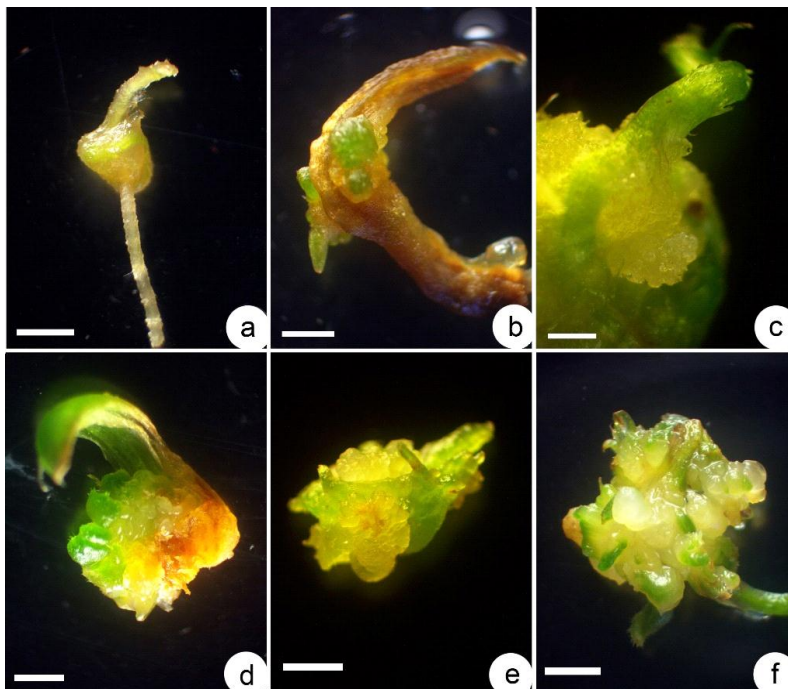
### Root formation

After 15 days, 67 to 75% of shoots induced roots when medium contained 0.1 mg/L NAA or IBA, or 100% if 0.1 mg/L of both these auxins were employed (Figure 3c and

Table 2). Control (no auxins) shoots did not induce roots within 15 days. However, after 30 days, 100% of shoots on any medium with an auxin formed roots (85% in the control) (Table 2).

### Callus induction and adventitious shoot induced from root explants

When BA, 2,4-D and NAA were used alone, almost no callus was induced from root explants, and only TDZ induced some expansion of the root explant and the induction of some callus. In all cases, adventitious shoot



**Figure 2.** Callus induction and differentiation of adventitious shoots from various explants (immature roots, young sheaths, base of shoots) of *Tripsacum laxum*. (a) expansion of immature root explants and induction of hard callus within 20 days on MS medium with 1.0 mg/L BA and 0.2 mg/L NAA. (b) expansion of immature root explants and induction of shoot buds within 30 days on MS medium with 1.0 mg/L TDZ and 0.2 mg/L NAA. (c, d) induction of friable callus and shoot buds within 30 days from young sheath explants on MS medium with 2.0 mg/L TDZ and 0.2 mg/L BA. (e, f) induction of friable callus and adventitious shoots buds from shoot base explants within 30 days on MS medium with 1.0 mg/L TDZ and 0.2 mg/L NAA. Bars = 3.0 mm.

Source: Authors

buds developed (Figure 2a). When thidiazuron (TDZ) and NAA were combined, the percentage of explants inducing callus increased to 13.3%, ultimately forming 2.2 adventitious shoot buds per explant after 30 days. Callus induction percentage (20% of explants) and number of adventitious shoot buds/explant (3.8) were largest on MS medium with 1.0 mg/L TDZ and 0.2 mg/L BA (Figure 2b and Table 3).

#### Callus and adventitious shoot buds induced from young sheath explants

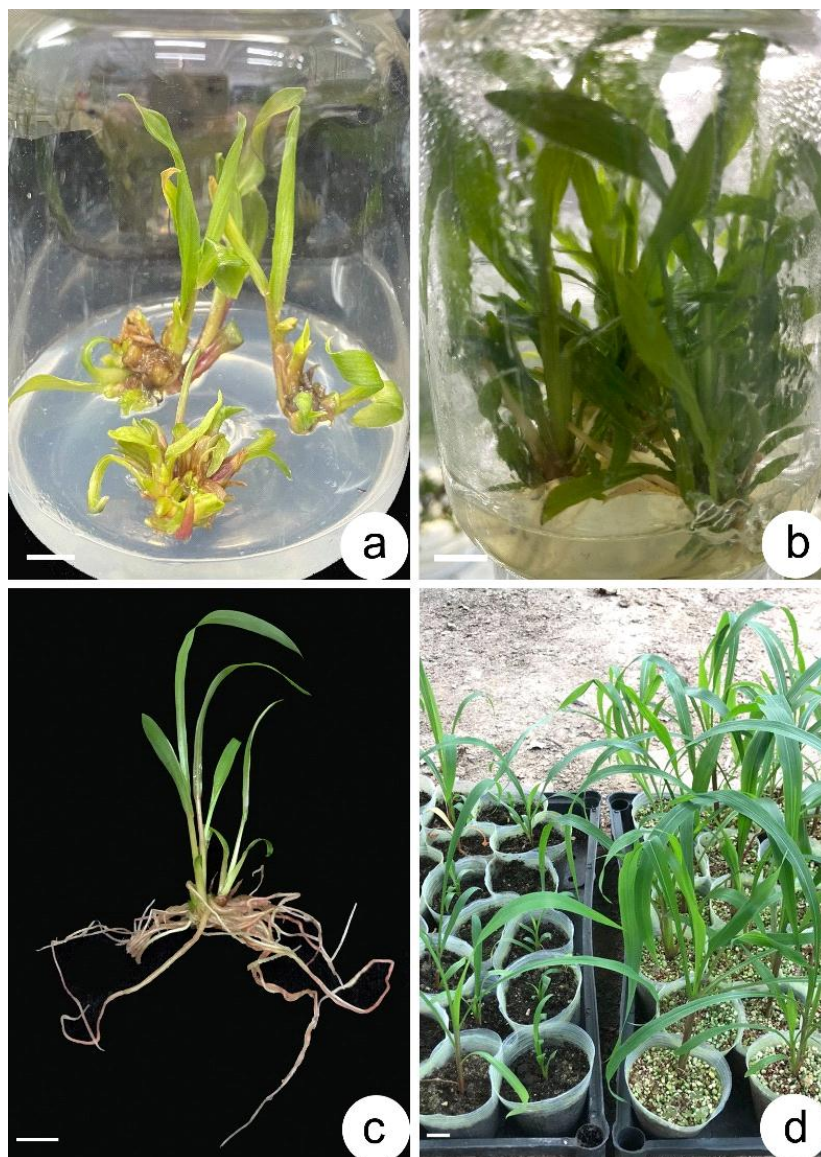
PGRs, when used alone, or a combination of BA/TDZ with NAA, could not induce callus from young sheath explants while TDZ with BA induced a low frequency (3.3-4.3%) of callus after 30 days. This callus was granular and beige-white (Figure 2c). Adventitious shoot buds were visible after 30 days. Optimal medium contained 1.0 mg/L TDZ and 0.2 mg/L BA, resulting in highest callus induction frequency (30.8%) most adventitious shoot buds/explant (4.6) (Figure 2d and Table 4).

#### Callus inducing from shoot basal meristem explants

When TDZ or 2,4-D were used alone, some hyperhydric pink callus was induced, but it was unable to differentiate, and eventually turned brown and died. BA did not induce callus, instead inducing adventitious shoots from callus. When 2,4-D was combined with BA and TDZ, they induced a low frequency of callus in 1 to 2% of explants after 30 days (Table 5). Milky white or yellow granular callus possessed a strong ability to develop adventitious shoot buds directly, especially the combination of 1.0 mg/L TDZ and 0.2 mg/L 2,4-D (9.2 adventitious shoot buds/explant) (Figure 2e and f), followed by 1.0 mg/L TDZ and 0.2 mg/L BA (5.3 adventitious shoot buds/explant) (Table 5).

#### Acclimatization and transplanting

Both treatments resulted in a high survival percentage, 99.3% in vermiculite: peat (1:3, v/v), and 96.7% in yellow mud and peat (1:1, v/v) (Table 6 and Figure 3d).



**Figure 3.** Shoot proliferation, rooting, transplanting and acclimatization of *in vitro*-derived *Tripsacum laxum* plantlets. (a) shoot proliferation on MS medium with 2.0 mg/L BA and 0.1 mg/L NAA for 25 days; (b) shoot proliferation on MS medium with 2.0 mg/L BA and 0.1 mg/L NAA for 45 days, with the formation of some small roots at the base of multiple shoots; (c) rooting of shoots on  $\frac{1}{2}$ MS medium with 0.2 mg/L IBA and 0.2 mg/L NAA for 30 days; (d) rooted plantlets were transferred to plastic pots containing peat and yellow mud (1:3, v/v) (left) and peat soil and vermiculite (3:1, v/v) (right) after 30 days, with more robust growth of plantlets on the right (also see Table 6). Bars = 1.0 cm  
Source: Authors

**Table 6.** *Tripsacum laxum* plantlet survival and height in different substrates after 30 days.

Substrate	Survival (%)	Plant height (cm)
Vermiculite: peat (1:3)	99.3 ± 0.7 <sup>a</sup>	25.3 ± 3.7 <sup>a</sup>
Yellow mud: peat (1:1)	96.7 ± 0.8 <sup>b</sup>	15.4 ± 2.5 <sup>b</sup>

Values represent means ± SD. Different letters within a column indicate significant differences according to Duncan's multiple range test ( $P \leq 0.05$ ). n = 30 per treatment.

Source: Authors

## DISCUSSION

The tissue culture of several species of the Gramineae employed various explants. For example, callus was induced from leaves in sugarcane on MS with 1.0 mg/L 2,4-D (Garcia et al., 2007), callus were induced from meristem tips in MS with 4.0  $\mu$ M BA and 40.0  $\mu$ M NAA (Lakshmanan et al., 2006; Tang et al., 2011), and callus were induced from sorghum immature embryos in MS with 2.0 mg/L 2,4-D (Assem et al., 2014). In the present experiment, peduncles were selected as explants because seed are not produced in nature (Dodds and Simmonds, 1946; Zhong et al., 2011). Since explants derived from field-grown plants are easy to become contaminated *in vitro* after inoculation on medium, despite surface disinfection, peduncles were selected as explants, reducing contamination-associated problems to about 3% in our initial trial.

Axillary shoot proliferation (that is, SPC) was enhanced in the presence of a cytokinin and NAA (Table 1), similar to the tissue culture of *L. repens*, another Gramineae plant (Xiong et al., 2021). In *T. dactyloides*, mature zygotic embryos were used to induce embryogenic callus cultures on MS medium with dicamba (10 or 20  $\mu$ M) and sucrose (3 or 6%), while plantlets were regenerated on PGR-free MS medium containing 2% sucrose (Furini and Jewell, 1991). In our study on *T. laxum*, only TDZ was able to induce callus from root explants, while the further addition of BA also stimulated shoot formation (Table 3). In dicotyledonous plants, the use of TDZ or BA are popular PGRs to induce shoot buds (Zhang et al., 2017; Liang et al., 2020), although TDZ might also induce somaclonal variation (Dewir et al., 2018). In monocotyledonous plants, 2,4-D has been used to induce callus and shoots from roots in sorghum (Mishra and Khurana, 2003), rice (Guo et al., 2018) and maize (Wang et al., 2021).

The base of leaf sheaths were used as explants to induce callus, although only TDZ combined with BA successfully induced callus, which differentiated into adventitious buds (Table 4). In sugarcane, in light, shoot induction from leaf explants was possible within 3 weeks on MS medium with 10 to 60  $\mu$ M NAA and 4 to 8  $\mu$ M BA (Lakshmanan et al., 2006), whereas in dark, callus was induced from stem segments or immature leaves when exposed to 4.5  $\mu$ M 2,4-D (Garcia et al., 2007).

## Conclusion

A protocol was developed for the regeneration of shoots from *T. laxum* peduncles via a direct shoot induction and shoot proliferation route and an indirect (callus-induced regeneration) route. The development of a protocol that will allow for the mass propagation of this species, will allow the resource allocation needs of this forage crop to be met, and allow for additional research such as genetic engineering to fortify abiotic stress tolerance.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

## ABBREVIATIONS

**2,4-D**, 2,4-Dichlorophenoxyacetic acid; **BA**, 6-benzyladenine; **IBA**, indole-3-butyric acid; **KIN**, kinetin; **MS medium**, Murashige and Skoog (1962) medium; **NAA**,  $\alpha$ -naphthaleneacetic acid; **PGR**, plant growth regulator; **SPC**, shoot proliferation coefficient; **TDZ**, thidiazuron.

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*Full Length Research Paper*

# **Prevalence of drug resistance and genetic characterization of *Mycobacterium tuberculosis* complex strains from pulmonary tuberculosis patients co-infected with malaria at Jamot Hospital in Yaoundé**

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**Tuberculosis (TB) is caused by the *Mycobacterium tuberculosis* complex (MTBC) and remains a major global public health concern. This study aimed to determine the prevalence of drug resistance and the genetic variation among MTBC population in pulmonary tuberculosis patients co-infected with Malaria at Jamot Hospital in Yaoundé-Cameroon. This was a 12 months cross-sectional study that enrolled 336 participants aged 15 years and above. Following sputum culture on solid media, drug resistance was detected using the proportion method and later confirmed by the Line Probe Assay. Isolates were further subjected to molecular characterization using spoligotyping. Amongst the 336 TB patients**

registered in this study, there were 17 (5.05%) TB-Malaria co-infected patients. Overall, in 25 (12.88%) patients the bacteria were resistant to at least one anti-TB drug, of which, 3 (1.54%) were co-infected with malaria. Multidrug-resistance (MDR) was observed in 2 cases (1.02%), 1 (0.51%) of which was in a TB-Malaria co-infected patient. *M. tuberculosis* was the only species identified. The T1 (60%) and the LAM10\_CAM (27.5%) families were the most prevalent genetic families both in TB-malaria co-infected and in mono-infected TB patients. The description of drug resistance prevalence and of the *M. tuberculosis* genetic diversity is expected to contribute to improving the TB case management in Cameroon.

**Key words:** Pulmonary tuberculosis, multidrug resistance, line probe assay, spoligotyping, genetic diversity, HJY, co-infection.

## INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*M. tuberculosis*), remains a major public health problem worldwide. According to reports from the World Health Organization (WHO) in 2019, 10 million new cases of tuberculosis were identified in the world, resulting in 1.2 million deaths. A number of 2.5 million new cases were and 377,000 deaths were reported in Africa in 2020 (WHO Report, 2020).

Tuberculosis also represents one of Cameroon's major threats to public health and a significant cause of preventable mortality in the adult population (NTCP, 2019). In 2018, 47,000 cases of tuberculosis were registered, with 7,700 cases of death (WHO Report, 2020).

On the other hand, malaria was responsible for 229 million cases in 2018 in the world, with 93% of cases occurring in Africa (WHO, 2019a). In 2017, malaria accounted for 24% of consultations and for 12.8% of deaths in Cameroon (National Malaria Control Strategic Plan in Cameroon, 2019). In co-infected patients, symptoms such as anemia, respiratory stress, cough, and fever are similar and delay diagnosis and initiation of treatment of either (Bahbahani and Al-Rashed, 2014). Malaria co-infection increases the mortality of TB patients. The disease increases the bacillary load, the hemozoin load, and disrupts the granuloma with altered responses (Colombatti et al., 2011).

Efforts to control TB were hindered in recent years due to the emergence of multidrug-resistant strains (MDR) to the first-line drugs. In 2018, the WHO identified 490,000 cases of MDR tuberculosis worldwide (WHO, 2019b). In 2016, the WHO estimated the number of rifampicin (RIF)-resistant MDR-TB (RR/MDR-TB) incident cases to be 1200 (1000-2200) corresponding to a prevalence rate of 6.8 (4.3-9.4) per 100,000 population in Cameroon (WHO, 2017). Further contribution to the increased death rate due to TB in the country was associated with the emergence and spread of drug resistant strains to nearly

all first line therapies (Kuaban et al., 2000a). RIF, isoniazid (INH), streptomycin (STM), and ethambutol (EMB) are components of first-line multidrug therapy in Cameroon (Soini and Musser, 2001). The rising prevalence of MDR strains has resulted in outbreaks and cases that are not only marginally treatable, but also often fatal. Following the awareness generated by the previous studies and the reorganization of the National TB Control Programme with focus on proper treatment methods, it has been necessary to reassess the levels of resistance to the main anti-TB drugs in the country. A systematic review analysis of drug resistance in Cameroon from 1998 to 2014 revealed the prevalence of drug resistance across some regions of the country. This analysis showed that in 2014, drug resistance reached 10.1% in the Centre region (Titanji and Assam, 2016). In addition, for 6 years, no further studies on resistance medication were conducted to get an idea of how the situation has evolved over the last few years. Furthermore, the long duration of culture (8 weeks), delays the detection of resistance and the proper management of TB patients. Molecular assays such as the line probe assay (LPA) have been recommended by WHO for the rapid detection of drug resistance to the most important first-line drugs (RIF and INH) (WHO, 2016).

Information on the *M. tuberculosis* complex (MTBC) genotypes is useful for understanding the spread and phylogeographic specificity of predominant clones, as MTBC lineages have differences in virulence, transmissibility, and capacity of acquiring drug-resistance conferring mutations (Coscolla et al., 2010). In this context, spoligotyping proves to be a very practical and reproducible tool. This method is based on PCR, which tests for the presence or absence of a set of target sequences in the Direct Repeat (DR) locus (Kamberbeek et al., 1997). The resulting genotype is a simple binary format, which has recently led to the construction of large

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databases, intended to facilitate the recognition and origin of a particular clinical isolate (Filliol et al., 2002). As in most countries with limited resources, the epidemiology of tuberculosis in Cameroon has so far largely consisted of reporting the number of cases detected and their demographic data. Thus, very little information is available on the strains of MTBC circulating in the country and more particularly in patients co-infected with TB and Malaria patients in Centre region. Indeed, for more than 5 years, no study has sought to determine the spoligotypes responsible for transmission in this region. The aim of this study was to describe the molecular drug resistance and assess the genetic diversity of isolates of the MTBC in TB/Malaria co-infected patients in the Centre region of Cameroon using spoligotyping.

## MATERIALS AND METHODS

This was a cross-sectional study with descriptive and analytical aims and lasted from April 2018 to March 2019. All consented smear-positive pulmonary tuberculosis patients, aged 15 years and above and consulting at the Jamot Hospital in Yaoundé were included in the study. Well-designed study questionnaires were used to capture clinical and epidemiological data. Sputum samples collected from patients were processed and confirmed for TB using microscopy in the hospital laboratory. Serology and malaria analyses were also performed under the same conditions.

Culture, drug susceptibility testing, molecular analysis and quality control (internal and external) were carried out at the Laboratory for Tuberculosis Research and Pharmacology (LTRP), located at the Biotechnology Center of the University of Yaoundé I.

### Sample processing

Samples were collected from each participant with productive cough on two consecutive days following standard procedures. Ziehl-Neelsen and/or auramine smears (Degommier, 1957) were performed at the recruitment site. Only the samples with the highest smear grade were transported in a cooler within one day to the LTRP for microscopic confirmation and culture. Each specimen was subjected to a decontamination step with cetylpyridinium chloride/NaCl. Anti-HIV antibodies were determined in whole blood by the immunochromatographic method the ALERE Determine kit, and all positive cases were confirmed using Oraquick. Each consenting participant received appropriate counseling prior to blood collection. Whole blood collected in the dry tube was used to perform the rapid diagnostic test for malaria using the Histidin Rich Protein2 (HRP2) kit specific to *Plasmodium falciparum* (Care Start™ Malaria pf Ag RDT). The results were confirmed by microscopy following staining of the slides with 5% Giemsa (Quakyi et al., 2000).

### Sputum culture, drug susceptibility test and identification

Following centrifugation of the sputum specimens, three to four drops of the suspended decontaminated sediment inoculate in 2 tubes, namely, the Löwenstein Jensen tube (LJ) without pyruvate and another one supplemented with 0.4% pyruvate. The inoculums were made on sloping media with inclination. The cultures were incubated at 37°C, followed by a weekly observation for growth and counting of colonies. The absence of a colony after 10 weeks of incubation was considered negative. For all positive cultures, drug

susceptibility testing was performed using the indirect proportion method on Löwenstein Jensen media at the following drug concentrations: INH (H1: 1 mg/ml and H2: 2 mg/ml), STM (S: 4 mg/ml), RIF (R: 40 mg/ml), EMB (E: 2 mg/ml). Drug resistance was defined as growth on a drug containing medium greater than or equal to 1% for INH and RIF, and 10% for STM and EMB (Canetti et al., 1963).

### DNA extraction

A loop full of mycobacterial colonies was scraped from Löwenstein-Jensen's media using a sampling loop, and introduced into eppendorf tubes containing Tris-EDTA (10 mM, 1 mM, pH 8) and heated for 30 min at 90°C. After centrifugation at 13,000 ×g, the supernatant was collected in a new tube and stored at -20°C until further use.

### Molecular detection of drug resistance by LPA

Molecular detection of drug resistance was performed with *MTB/DRplus* (Hain Diagnostics), an LPA that targets wild-type regions and mutations in *rpoB* codons 516, 526 and 531 associated with RIF resistance; and in *katG* codon 315 and *inhA* positions 16, associated with INH resistance (Molina-Moya et al., 2015). TB Resistance assay was performed following the manufacturer's instructions. Hybridization and detection were performed with LPA (*MTB/DRplus* version 1.0 kit). The presence of all wild-type hybridization bands and absence of mutation bands indicated a susceptibility to the drug considered. The absence of at least one wild-type hybridization band and/or the presence of any mutation band indicated resistance to the drug considered. The presence of all wild-type hybridization bands in combination with a mutation band in a target gene indicates heteroresistance, a combination of both susceptible and resistant *M. tuberculosis*.

### Genotyping of MTBC by spoligotyping

All the isolates were genotyped with a commercial spoligotyping kit (Isogen Bioscience, BV Maarsen, The Netherlands) as described previously by Kamerbeek et al. (1997). Briefly, the DR region of the genome of the tuberculosis isolates was amplified using the primers DRa, 5'-GGTTTTGGGTCTGACGAC-3' (biotinylated end 5') and DRb, 5'-CCGA-GAGGGGACGGAAAC-3'. PCR products were hybridized with a set of 43 spacer oligonucleotides covalently linked to the spoligotyping membrane (Isogen Life Sciences, The Netherlands) according to the manufacturer's instructions. The hybridized PCR products were then incubated with streptavidin-peroxidase conjugate and the membrane exposed to chemiluminescence (Amersham ECL Direct™ Nucleic Acid Detection and Labeling System, GE Healthcare Limited, UK). The X-ray film was developed using standard photochemical procedures after 20 min exposure in the darkroom. DNA extracts from *M. tuberculosis* H37Rv and *M. bovis* BCG were used as controls.

### Data analysis

The sample size was calculated by the formula  $n = z^2 \times p(1-p) / m^2$ . With  $p = 0.27$ ,  $z = 1.96$ ,  $m =$  margin of error (5%). By calculation we have 303 TB patients, and we have maximized the sample size to 336 patients.

Socio-demographic and clinical data obtained through questionnaires and the results of laboratory tests were entered, cleaned and analyzed using the statistical software for social sciences (SPSS) version 22.1, data record files. Spoligotype

patterns in binary format were entered into a Microsoft 2016 Excel sheet, and compared to the SpolDB4 database using *MIRU-VNTRplus* software. The Hunter Gaston Discriminant Index (HGDI) was used to calculate the discriminating power of the spoligotyping method. Fisher's Chi-square or Exact Test was used to assess the correlation between epidemiological data and isolate genotypes. P-values less than 0.05 were considered statistically significant.

### Ethical consideration

This study complied with the standards of the National Research Ethics Committee for Human Health (CNERSH) N°2018/01/970/CE//SP. Administrative authorization was obtained from Jamot Hospital (N°: 00001478/L/MINSANTE/SG/DHJY). All study procedures were carefully explained before and during the study. Written, informed and signed consent (provided in French and English languages) was obtained from each enrolled patient who incurred no cost for sample processing. All drug susceptibility test results were reported to the respective health facilities for further management of the patients. Furthermore, the confirmed MDR-TB case identified in this study was referred to the MDR-TB treatment center for further management.

## RESULTS

### Socio-demographic characteristics of the subjects of study

Out of a total of 336 tuberculosis patients included in this study, 215 (63.98%) were males, while 121 (36.01%) were females, with a male to female ratio of 1.77:1. The average age of the patients was (35.16 ± 14.04 years), with minimum age of 15 years. Regarding the marital status and level of education, 222 (66.07%) were singles, 94 (27.97%) were married, 14 (4.16%) were widowed and 6 (1.78%) were divorced, while 17 (5.05%) were out of school. On the level of education, 77 patients (22.91%) had a primary level, 194 (57.73%) had a secondary level and 48 (14.28%) had university level studies. Regarding the HIV status of the study participants, 99 (29.46%) tested positive for HIV.

A total of, 17/336 (5.05%) cases of TB-Malaria co-infection were found with a prevalence of co-infection comparable in males 9/17 (52.94%) and females 8/17 (47.05%). The most affected were found the patients aged between 30 and 50 years 8/17 (47.05%). Most of the co-infected individuals were single 13/17 (76.47%), had higher educational training 12/17 (70.58%), worked in the informal sector 14/17 (82.35%) and resided in the urban area 13/17 (76.47%). Table 1 presents the socio-demographic characteristics of the patients in this study.

### Results of tuberculous mycobacteria cultures and resistance pattern to first line anti-TB drugs

The study of the distribution of cultures of *M. tuberculosis* showed that out of the 308 sputum samples cultured, 194 (62.98%) were positive cultures. Of these, there were 12

positives cultures among co-infected patients. Drug sensitivity test to the first line anti-TB drugs INH, RIF, EMB and STM was carried out on a total of 308 *M. tuberculosis* isolates. The highest proportion of mono resistance was observed against STM 12/25 (7.2%) followed by EMB 7/25 (3.6%) and INH 2/25 (1.03%). There was no observed mono-resistance to RIF (0 %). Poly-drug resistance among new TB patients was observed with INH + EMB only 1/25 (0.51%), INH + STM only 1/25 (0.51%) and INH+RIF+EMB only 1/25 (0.51%). Two isolates 2/25 (1.02%) were resistant to RIF and INH (MDR). Initial and acquired resistance was 11/25 (7.17%) and 14/25 (5.67%), respectively. Three co-infected TB/Malaria participants 3/25 (1.54%) were resistant to EMB. Only one case of MDR INH+RIF+EMB 1/25 (0.51%) was found among co-infected patients (Table 2).

### Distribution of the different genetic families

A total of 9 genetic profiles were identified within 4 large genetic families among the 80 isolates. Thus, 22/80 (27.5%) of the isolates were from the LAM10\_CAM family, 2/80 (2.5%) from the Harlem family, 1/80 (1.25%) from the Uganda family, 48/80 (60%) from the T1 Ghana family and 7/80 (8.75%) cases were not identified by the database as indicated in Table 3.

### Predominant spoligotypes

Sixteen (16) spoligotypes were identified amongst which, nine (9) represented Shared Types (ST) already known and already listed in the SpolDB4 database. The remaining seven (7) spoligotypes were identified for the first time. Among these Shared Types, the ST 53 (Ghana family) member of the T1 family, and the ST 61 member of the LAM10\_CAM family were, respectively represented by 47.5 and 26.25% shown in Table 4.

### Distribution of spoligotypes (Share type) according to patient types (mono-infected TB and co-infected TB-malaria)

These results showed that the co-infected participants were infected with strains belonging to the LAM10\_CAM families and the T1 family. In co-infected participants, the LAM10\_CAM family was represented exclusively by the ST61 spoligotype. This spoligotype was also dominant in the mono-infected participants. However, the presence of a strain with the spoligotype represented by ST838 was also noted. Within the T1 family, the presence of 2 spoligotypes was observed in the co-infected patients and represented by: ST53 and ST123. Moreover, in the mono-infected participants, apart from these 2 spoligotypes, the spoligotypes represented by ST205, ST774 and ST498 were also recalled. In view of these

**Table 1.** Sociodemographic characteristics of the study population.

Characteristics	Total number	Percentage	TB/Malaria Co-infected	Percentage
<b>Sex</b>				
Female	121	36.01	8	47.05
Male	215	63.98	9	52.94
Total	336	100	17	100
<b>Age (years)</b>				
<20	28	8.33	3	17.64
21-30	112	33.33	4	23.52
31-40	96	28.57	3	17.64
41-50	49	14.58	5	29.41
51-60	28	8.33	1	5.88
61-70	18	5.35	1	5.88
71 and above	5	1.48	0	0
Total	336	100	17	100
<b>Marital status</b>				
Singles	222	66.07	13	76.64
Married	94	27.97	0	0
Divorced	6	1.78	3	17.64
Widowers	14	4.16	1	5.88
Total	336	100	17	100
<b>School level</b>				
Out of school	17	5.05	1	5.88
Primary	77	22.91	3	17.64
Secondary	194	57.73	12	70.58
University	48	14.28	1	5.88
Total	336	100	17	100
<b>Type of infection</b>				
TB	237	65.49	-	-
TB-HIV	99	29.46	-	-
TB/Malaria	17	5.05	-	-
Total	336	100	-	-

Source: Microsoft word office 2010

results, co-infected participants showed low genetic diversity compared to mono-infected participants (Table 5).

#### **Predominant families and transmission rate in TB/Malaria co-infected patients and mono-infected TB**

The genetic profile was generated by the SpolDB4 database and SITVIT WEB, and allowed to calculate the transmission rate (88.75%) and the Hunter Gaston Discriminating Power Index (HGDI) of 64.6%. Overall, this transmission rate implies that 88.75% of the strains

are involved in a recent transmission chain. Thus, the disease reactivation rate was 11.25%. In co-infected patients the rate of transmission of the disease was lower (83.3%) and the rate of reactivation higher (16.07%). Only 2 genotype families were found in cases of coinfection, namely the LAM10\_CAM and T1 family (Table 6).

#### **Correlation between drug resistance and genetic diversity of *M. tuberculosis* strains**

A statistically significant difference was found between the T1 family and streptomycin monoresistance ( $p=0.046$ ).

**Table 2.** Resistance pattern to first line anti-TB drugs among new smear positive pulmonary TB and co-infected patients at Jamot Hospital in Yaoundé Cameroon.

Variable	Number/Frequency of appearance (%)	TB/Malaria co-infected patients/Frequency of appearance (%)
Resistance to only one drug		
INH only	2 (1.03)	-
RIF only	-	-
STM only	12 (7.2)	-
EMB only	7 (4.2)	2 (1.02)
Poly-resistance	-	-
INH+EMB	1 (0.51)	-
INH+STM	1 (0.51)	-
INH+RIF+EMB (MDR)	1 (0.51)	1 (0.51)
INH+RIF(MDR)	1 (0.51)	-
MDR Global	2 (1.02)	-
Total	25 (12.88)	3/25 (1.54)
<b>Type of resistance</b>		
Initial resistance	11 (5.67)	1 (0.51)
Acquired resistance	14 (7.17)	2 (1.03)
Total	25	3

Source: Microsoft word office 2010

**Table 3.** Distribution of the different genetic families identified from 80 isolates of the *Mycobacterium tuberculosis* complex.

Genetic family	No. of Isolates	Frequency of appearance (%)
LAM10_CAM	22	27.5
Haarlem 3	2	2.5
Uganda 1	1	1.25
T1	48	60
Not identified	7	8.75

Source: Microsoft word office 2010

These 12 cases of streptomycin monoresistance were identified mainly within ST53 suggesting that particular attention should be paid to this spoligotype. The T1 family presented a case of MDR among the 2 cases of MDR that we observed in this study. The only case of MDR among the co-infected patients was also resistant to ethambutol and belonged to the LAM10\_CAM family (Table 7).

## DISCUSSION

The overall objective of this study was to determine the prevalence of drug resistance to anti-tuberculosis drugs and genotyping the isolates of MTBC among TB-Malaria co-infected patients at Jamot Hospital in Yaoundé (Cameroon).

A total of 336 patients with microscopically positive pulmonary tuberculosis were enrolled. The majority of the

patients were males, 215 (63.98%), which corroborates the work of Pokam, Kuaban, and Assam Assam (Pokam et al., 2020; Kuaban et al., 2020; Assam Assam et al., 2011). In this study, the prevalence of malaria among TB patients was low 17/336 (5.05%). Similar studies have reported a low prevalence of TB/Malaria co-infection. In Cameroon, Anyangwe (Anyangwe, 2016) found a co-infection prevalence of 1.5%. A similar study by Range (Range et al., 2007) in Tanzania found a coinfection prevalence of 4.3%. Similarly, Baluku's study in 2019 in Uganda also found a low coinfection prevalence of 2.2% (Baluku et al., 2019). This low prevalence might be due to the fact that tuberculosis results in the production of interferon gamma (IFN- $\gamma$ ), tumor necrosis factor alpha (TNF- $\alpha$ ), and humoral factors that are protective of malaria (Page et al., 2005; Murphy, 1980).

One of the major problems responsible for treatment failure in tuberculosis patients is the emergence of strains resistant to anti-tuberculosis drugs. In the present study,

**Table 4.** Distribution of Shared Type identified from the collection of 80 isolates of the *Mycobacterium tuberculosis* complex.

Genotype family	Share type	Spoligotypes	No. of isolate(s)	Frequency (%)
LAM10_CAM	61		21	26.25
	838		1	1.25
T1 family	53		38	47.5
	123		6	7.5
	205		1	1.25
	774		1	1.25
Uganda	498		2	2.5
	237		1	1.25
Haarlem3	50		2	2.5
Not identified	-			
	-			
	-		7	8.75
	-			

Source: Microsoft word office 2010

**Table 5.** Distribution of predominant spoligotypes (Share type) according to the types of patients: co-infected TB-malaria and mono-infected TB.

Genotypes family	Co-infected TB/Malaria		Mono-infected	
	ST	proportion	ST	Proportion
LAM10_CAM	ST61	4	ST61	17
	ST838	-	ST838	1
T1 family	ST53	4	ST53	34
	ST123	4	ST123	2
	ST205	-	ST205	1
	ST774	-	ST774	1
	ST498	-	ST498	2
	N=12		N=58	

Source: Microsoft word office 2010

the overall rate of resistance (to one or several drugs) was 12.88% corresponding to the global resistance. This resistance rate is close to that reported earlier (13.93%) by Tchatchouang et al. (2015) in the Centre region of Cameroon. This rate is slightly higher than those found by Assam et al. (2011) and Sidze et al. (2014), which were 8.1 and 10.1%, respectively. However, high rates have been found by several authors in Africa namely: 23% in Ethiopia, 46.7% in Nigeria (Seyoum et al., 2014; Otokunefor et al., 2018). The primary resistance rate in this work was 5.67% while the secondary resistance rate was 7.17%. A study conducted in the Centre Region in 1998 showed initial and acquired resistance rates to be 31.8 and 55.8%, respectively, another study conducted in 2011 had also shown initial and acquired resistance of

7.35 and 16.66% (Bercion and Kuaban, 1998; Assam et al., 2011). This drop could be the fall-out of the reorganization of the National TB Control Programme, which emphasises the implementation of the Directly Observed Treatment Strategy (DOTs). The highest resistance rates to a drug were observed with streptomycin (7.2%). A similar study in the Centre region of Cameroon in 2015 showed the frequency of resistance to streptomycin was 8.86% (Tchatchouang et al., 2015). Moreover, another study in Mozambique in 2017 showed an overall resistance to streptomycin of 4% (Valencia et al., 2017). The high prevalence to streptomycin may be due to its use in the treatment for other infections in Cameroon. The lowest resistance rate was found with isoniazid (1.02%). Some work carried out in the Centre



**Table 6.** Summary of predominant families involved in TB-Malaria co-infection and transmission percentage.

Genotype	LAM10_CAM		Family T1		Percentage of transmission	
	Frequency	Proportion (%)	Frequency	Proportion (%)	Rate of recent transmission	Rate of reactivation
Mono-TB	18	81.81	40	83.33	96.25	3.50
TB/Malaria Co-infected	4	18.18	8	16.66	83.3	16.07
P-values	0.402		0.634			

Source: Microsoft word office 2010

**Table 7.** Relationship between *M.tuberculosis* families and resistance to anti-TB drugs.

Resistance to:	Type of infections	Anti-TB drug	Families	Spoligotyping (Share type)	No. of isolate(s)	P-value		
One anti-TB drug	Mono-infected TB	INH	H3	ST50	1	0.644		
			LAM10_CAM	ST61	1	0.066		
		RIF	-	-	-	-	-	
			Orphan	-	-	2	-	
		STM	T1		ST205 (1)			
					ST53 (1)	12	0.046	
					ST53 (10)			
		EMB	T1	LAM10_CAM	LAM10_CAM	ST61	1	0.893
					Orphan	-	1	-
					ST498 (1)			
Co-infected TB-Malaria	EMB	T1		ST123 (1)				
				ST53 (2)	6	0.587		
				ST53 (2)				
Two anti-TB drugs	Mono-infected TB	STM-EMB	LAM10_CAM	ST61	1	0.217		
		INH-RIF	T1	ST53	1	0.241		
Three anti-TB drug	TB-Malaria Co-infected	INH-RIF-EMB	LAM10_CAM	ST61	1	0.257		

Source: Microsoft word office 2010

region (Jamot hospital and Mbalmayo district hospital) has shown high rates of isoniazid resistance of 5.06 and 4.7%, respectively

(Tchatchouang et al., 2015). Despite the low rate of resistance to isoniazid in this study, it remains permanent, the constant presence of resistance

to isoniazid can be justified by its long period of use in the treatment of tuberculosis (Assam et al., 2011). A more serious aspect of the TB drug

problem is when the infecting organism is resistant to both INH and RIF, referred to as MDR-TB (Canetti et al., 1963). Under this condition, the duration of treatment is prolonged from 6 to 18-24 months, and the cure rate could decrease from nearly 100% to less than 60%. This makes the treatment of MDR cases particularly challenging (Warren et al., 2006). MDR-TB was demonstrated in 2 patients (1.02%). Kuaban in 2000 noted an MDR rate of 4.1% in the West region (Kuaban et al., 2000b), Assam Assam noted an MDR rate of 6.6 % in the same region, but the Centre region in noted an MDR of 1.1% (Assam et al., 2011; Sidze et al., 2014). The lower resistance rates for the Centre region in the present study could be accounted for, at least in part, by the fact that the region is the seat of the capital city of Cameroon, Yaoundé where better health care facilities may be found.

Molecular characterization of *MTBC* strains was performed using the spoligotyping technique. In this study, *M. tuberculosis* was the only species found. This result is similar to that obtained by Assam (2020) in western Cameroon which showed that 100 % of the strains belonged to the species *M. tuberculosis* (Assam et al., 2020). However, this result was slightly different from the 97.65, 98.8 and 97.3% obtained the rate of *M. tuberculosis*, respectively by Kamgue (2013), Assam Assam et al., (2013) and Onana et al., (2018).

However, mass BCG vaccination applied for decades could shape the structure of the *M. tuberculosis* population (Hermans et al., 1995). In addition, the same studies mentioned earlier reported low prevalence of *M. africanum* in the following proportions 2.03, 1.2 and 2.74% (Kamgue et al., 2013; Assam et al., 2013; Onana et al., 2018). This strong presence of *M. tuberculosis* and the virtual absence of *M. africanum* does not corroborate with the studies carried out in 1970 which showed that the majority of cases of TB were caused by *M. africanum* (56%) (Huet et al., 1971). Three decades after, the study conducted by Niobe-Eyangoh et al. (2003) suggests that the downward trend observed over the past decades probably reflects a real regression of *M. africanum* (56 to 9%) as an etiologic agent of tuberculosis in Cameroon (Niobe-Eyangoh et al., 2003). This drop in *M. africanum* for the benefit of *M. tuberculosis* has been confirmed by the studies of Simon et al. (1989), Ledru et al. (1996) and Bourahima et al. (2022) which have shown a regression of *M. africanum* in Burkina-Faso and in Mali. This study reveals the highly diverse *M. tuberculosis* population structure, it confirms a predominance of the Cameroon lineage in the Centre region of Cameroon and the disappearance of *M. africanum* in Cameroon. The causes of this regression are not known. However, the treatment protocol applied to the patients could play an essential role in this selection (Vekemans et al., 1999). Likewise, the *M. bovis* species was not found in this study. This result corroborates with work carried out in Cameroon and Burkina-Faso which shows the low prevalence or the absence of *M. Bovis* in humans (Niobe-Eyangoh et al.,

2003; Ledru et al., 1996). This low prevalence can be explained by the fact that the majority of TB cases due to the *M. bovis* species are associated with extrapulmonary TB cases. In this study, we considered only pulmonary tuberculosis. In addition, cases of TB due to *M. bovis* are more frequent in rural areas (Vekemans et al., 1999), but the study was conducted in an urban area. Similarly, the culinary practices that are popular in Africa, consisting in cooking meat well before consumption can also explain the low prevalence of *M. bovis*. The factors that may explain the adaptability of *M. tuberculosis* to a particular area are poorly understood.

The search for the genetic profile of our 80 isolates was done using the database (SpolDB4) and SITVIT WEB and this enabled us to identify 4 major spoligotypes families namely LAM10\_CAM (27.5%), Haarlem (2.5%), T1 (60%), Uganda (1.25%) and unidentified cases (8.75%). The major family in this study is the T1 family (60%), contrary to those obtained (27%) by Assam et al. (2013) in the Centre region. The same is true of the 31.66% obtained by the latter (2020) in the western region and 31.84% obtained by Bourahima (Assam Assam et al., 2020; Bourahima et al., 2022). The high prevalence of the T1 family in particular in this study is intriguing and should be followed in studies based on a large population. Of these 60%, the most represented clade was Shared Type 53 or Ghana family with a proportion of (47.5%) compared to other isolates. However, we noticed a strong propensity of ST 53 (47.5%) compared to 11.3% obtained by Pokam in Littoral region of Cameroon in 2020 and 14.7% obtained by Bourahima in Centre of Bamako (Pokam et al., 2020; Bourahima et al., 2022). This therefore implies a strong infiltration of the Ghana family during the last 18 years in Cameroon, probably due to the phenomenon of cross-border migration of strains. The second family mainly represented was the LAM10\_CAM family (28.20%) which corroborates with the 34% obtained by Assam et al. (2013), but was different from the 43.33% obtained by Assam et al. (2020) and 46.73% obtained by Niobe-Eyangoh et al. (2003) as well as 51.01% by Kamgue et al. (2013). Beyond our borders, 63% obtained by Molina-Moya et al. (2018) in Nigeria and 45.1% obtained by Bourahima et al. (2022) in Mali. The strong regression of the LAM10\_CAM family in this study in favor of the T1 family is intriguing and should be followed in studies based on a larger population. Within the LAM10\_CAM family, a diversity was found and the Share Type 61 (26.25%) was predominant within this family. This result corroborates with those of Koro Koro et al. (2013) who showed a predominance of ST 61 within the LAM10\_CAM family.

## Conclusion

The description of drug resistance prevalence and of the *Mycobacterium tuberculosis* genetic diversity is expected

to contribute to improving the TB case management in Cameroon.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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*Full Length Research Paper*

# Effects of 2-chlorophenol and 2,4-dichlorophenol on mitotic chromosomes and nucleic acid content using *Allium cepa* and *Vicia faba* assays

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Chlorophenols are compounds used as intermediaries in manufacturing agricultural pesticides. Despite the efficiency on their targets, they are considered as environmental pollutant substances. This study assessed two chlorophenol substances, 2-chlorophenol and 2,4-dichlorophenol (2-CP, 2,4-DCP) for their cytogenetic, genotoxic, and nucleic acid content affects using *Allium cepa* and *Vicia faba* assay. In *A. cepa*, 2-CP low dosages (0.625%) increased the mitotic index (MI), while high dosages (1.25 and 2.5%) decreased it; the high dosage (2.5%) increased the chromosomal aberration frequency (CAF). Treatment with 2,4-DCP showed that 2.6% was the most affected treatment, it decreased MI and increased CAF. In *V. faba*, both 2-CP and 2,4-DCP decreased MI and increased CAF. Types of chromosome anomalies scored after treatment with different concentrations were chromosome stickiness, disturbance, lagging chromosomes, anaphase and telophase bridge, and micronuclei. The contents of DNA and RNA in *A. cepa* were increased by 2-CP and 2,4-DCP, and decreased by the half dosage of 2-CP (1.25%); the RNA content varied directly proportional to DNA, while in *V. faba*, all tested concentrations of chlorophenols increased nucleic acids contents, and 1.3% was significantly affected. The results showed that the two substances have cytogenetic, genotoxic impact, and also have influence on the nucleic acid contents in both plants.

**Key words:** Cytogenetic, genotoxic, chlorophenols, *Allium cepa*, *Vicia faba*.

## INTRODUCTION

Chlorophenols act as intermediaries in manufacturing agricultural chemicals, biocides, and dyes (Igbinosa et al., 2013). Although agricultural chemicals significantly affect disease control, they can also cause considerable environmental and health difficulties from production through disposal. Chlorophenols are considered

environmental pollutants due to chemical and pharmaceutical industrial activities (Jensen, 1996; Czaplicka, 2004; Michalowicz and Duda, 2007; Igbinosa et al., 2013; Adeola, 2018). They enter the environment through various sources, such as industrial waste, pesticides, or by degrading complex chlorinated

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hydrocarbons (Igbiosa et al., 2013). In Finland, people's exposure to contaminated drinking water with chlorophenols caused digestive tract infection, asthma, depression, and morbidity (Lampi et al., 2000). Chen et al. (2004) proposed that chlorophenols have cytotoxic and cell death mechanisms. Atuanya and Onuoha (2018) suggested continues monitoring of agricultural farmlands for the continuous exposure to pesticides that led to food contamination. Treatment with different chlorophenols caused various problems in cells, such as disturbance in the cell cycle, chromosome aberrations, and genetic material damage in other tested living systems. Researchers have documented these cases. Küçük and Liman (2018) found that treatment with different concentrations of 2-chlorophenol (2-CP) slightly decreased mitotic index (MI) and several types of chromosomal aberrations, such as disturbance, chromosome laggards, stickiness, and bridges on *Allium cepa* root tip cells.

Küçük and Liman (2018) discussed the effects of chlorophenols on genetic material and found that they could oxidize DNA bases in human lymphocytes and that pyrimidine bases were more strongly oxidized compared to purine ones. Also, the effects are not only confined to the parent material, but also to the transformation products. Benfeito et al. (2014) found that chlorophenoxy herbicide transformation products had inhibitory activity on DNA compared with the parent herbicides. Küçük and Liman (2018) concluded that 2-CP affected DNA at significant levels. Several studies have shown that chlorophenols affect DNA (Dimitroy and Gadeva, 2006; Tingting et al., 2017).

The most significant commercial chlorophenols are 2,4-DCP (Czaplcka, 2004), pentachlorophenol (PCP) (Michalowicz and Duba, 2007), 2,4,5-trichlorophenol (2,4,5-TCP) (Jensen, 1996), 2-CP (Balcke et al., 2008), and 4-CP (Abhilash and Singh, 2008).

2-CP is a chlorinated organic class used as an intermediate for synthesizing higher chlorinated congeners, certain dyes, herbicides, fungicides, and plastics (Küçük and Liman, 2018). Previous studies have confirmed the harmful effect of 2-CP. Dimitris et al. (2016) tested the impact of 2-CP on bacteria, fish, and human cells. They found that treatments can induce dose-dependent toxic and genotoxic effects.

2,4-DCP is a colorless crystalline solid, a chlorinated phenol derivative used as an intermediate for herbicide preparation of 2,4-dichlorophenoxyacetic acid. Previous studies of 2,4-DCP and its derivatives have revealed that while killing harmful organisms, it will upset the ecosystem and produce undesirable changes in higher organisms (Ajay and Sarbhoy, 1988); its destructive and toxic substance can be referred for their easy skin and epithelium penetration, thereby causing damage and necrosis (Michalowicz and Duda, 2007).

Higher plant assays are used in monitoring and detecting cytogenetic and mutagenic effects and have

been recognized as excellent genetic models. The *A. cepa* assay is an efficient test for chemical screening for genotoxicity of environmental contaminants and has been widely used in studying genotoxicity of many pesticides, revealing that these compounds can induce chromosomal aberrations in root meristems of *A. cepa* (Ma et al., 1994; Fernandes et al., 2007).

*Vicia faba* bioassay has been used to study DNA damage and chromosomal and nuclear aberrations. The main benefits of using *V. faba* are accessibility during the year, easy to grow and handle, cell division frequency is fast, easy chromosomes scoring, less expensive, and more sensitive (Iqbal, 2016).

This study assesses 2-CP and 2,4-DPC effects on MI and CAF, and their impact on nucleic acid content using *A. cepa* and *V. faba* assay.

## MATERIALS AND METHODS

### Sample preparation

(1) *A. cepa* bulbs were obtained from the local markets. Bulbs of *A. cepa* outer loose crusts and old roots were disposed, the running water with the help of a brush was used to remove the remaining sands particles to expose the root tips for different treatments.

(2) *V. faba* seeds were obtained from the local market. They were sterilized using sodium hypochlorite for 5 min, then presoaked for 24 h in distilled water.

### Tested materials

(1) 2-CP of three different concentrations was prepared (0.625, 1.25, and 2.5%), and distilled water was used to prepare the solutions.

(2) 2,4-DCP of three different concentrations was also prepared (0.65, 1.3, and 2.6%), and distilled water was used to prepare the solutions.

### Sample treatments

(1) *A. cepa* bulbs' root zone of the onion bulb was submerged in a cup filled with distilled water until the roots reached 2 to 3 cm long; then, they were transferred to different concentrations of each substance, freshly prepared for different periods (8, 16, and 24 h).

(2) The soaked seeds of *V. faba* were germinated in distilled water until the secondary roots reached 1 to 2 cm and were transferred to different tested concentrations of each substance, freshly prepared for different periods (8, 16, and 24 h).

The treated root tips of *A. cepa* and *V. faba* were cut and fixed in freshly prepared 3:1 (v/v) alcohol: glacial acetic acid for 24 h. For cytological preparations, the treated root tips were hydrolyzed in 1 N HCL 60°C for 7 min and were washed with distilled water and stained with 1% acetocarmine; five temporary slides were prepared using the squash technique, and two root tips on each slide were examined for the tested material effects on MI. The same slides were analyzed for the types and frequencies of chromosomal abnormalities.

### Nucleic acids (DNA, RNA) isolation and measurement

Genetic material isolation was applied following the instructions of

Hi Pura TM (Plant DNA isolation (CTAB Method) kit). The concentrations of isolated nucleic acids were measured by IMPLN Nano Photometer® instruments, version NPOS 3.1c build13220.

### Slides scoring and data analysis

**Studying the slides:** Slides were viewed under a light microscope (Phenix P H 50 DB047VU) using 40X objective lens immersion.

**Mitotic index (MI):** On one slide for each treatment, a total of 2000 cells were scored. The MI was expressed as the number of dividing cells per total cells scored.

**Cytotoxicity:** The MI of the treated cells for each dose of each material was compared with that of the negative control.

**Genotoxicity test:** Chromosomal aberrations per dose of each material were examined; the most representative ones for each structural abnormality were photographed using (Phenix micro-Image Analyzer Software 2008 En V2).

### Statistical analysis

The percentages of MI and CAF of each tested dose were compared with those of the negative control using the SPSS 16.0 for Windows statistical package. A two-way analysis of variance (ANOVA) was used to determine the significance of the difference at  $P \leq 0.05$ .

## RESULTS

### MI and CAF

Tables 1 and 2 show 2-CP and 2,4-DCP effects on the MI in meristem cells of *A. cepa* and *V. faba* root tips.

Table 1 and Figure 1 show that 2-CP tested concentrations affected the MI of *A. cepa* root tip cells compared with the control. Treatment with 0.625 and 2.5% for 8 h increased the MI, and it was insignificant at  $p > 0.05$ , while other treatments decreased it; treatment with 2.5% for 16 h was significant at  $p < 0.05$ .

2,4-DCP also affected the MI in Figure 2 and treatment with 0.65 and 2.6% decreased the MI after 8-h exposure, and it was significant at  $p < 0.05$ , while treatment with 1.3% increased it and was insignificant at  $p > 0.05$ ; also, treatment with 0.65 and 2.6% for 16 h decreased the MI of *A. cepa* root tip cells, and it was significant at  $p < 0.05$ .

Table 2 and Figure 3 show the 2-CP effect on the MI on root tip cells of *V. faba*, different tested concentrations for different periods decreased the MI compared with the control; treatment with 0.625% increased the MI after 16 h exposure, and it was insignificant at  $p > 0.5$ , while treatment with 1.25% for 16 h and 2.5% for 24 h decreased the MI significantly at  $p < 0.05$ .

Figure 4 shows that the treatment with 0.65% of 2,4-DCP for 16 and 24 h increased the MI with 9.8 and 9, respectively, compared with the control (7, 8), and it was insignificant at  $p > 0.5$ ; also, treatment with 1.3% for 8 and 16 h increased the MI with 10 and 8.3, respectively, compared with the control (8.5, 7), and it was insignificant

at  $p > 0.5$ ; treatment with higher concentration of 2.6% of 2,4-DCP for 16 h increased the MI insignificantly at  $p > 0.5$ . It decreased the MI with 4.6 compared with the control eight after 24 h, and it was significant at  $p < 0.5$ .

The tested materials affected the CAF compared with the control. Table 1 and Figure 5 show that treatment with different concentrations of 2-CP increased the CAF with 2.5% after 8 h, and 1.25 and 2.5% after 24 h, while with 0.625 and 2.5% after 16-h exposure, and they were insignificant at  $p > 0.05$ .

Figure 6 shows the effect of treatment with 2,4-DCP for different periods. It increased the CAF compared with the control in *A. cepa* root tip cells, and treatment with 0.65% for 24 h and

treatment with 2.5% for 16 h were significant at  $p < 0.05$ .

In *V. faba*, Table 2 and Figure 7 show that CAF increased after treatment with different concentrations for different periods compared with the control, treatment with 0.625% increased the CAF, also 0.13% after 8-h exposure and was significant at  $p < 0.05$ ; treatment with 1.25% for 16 and 24 h increased the CAF with 0.1 each, and it was significant at  $P < 0.05$ .

Figure 8 shows that treatment with different concentrations of 2,4-DCP affected the CAF. All the tested concentrations increased the CAF, and it was significant at  $p < 0.05$  compared with the control.

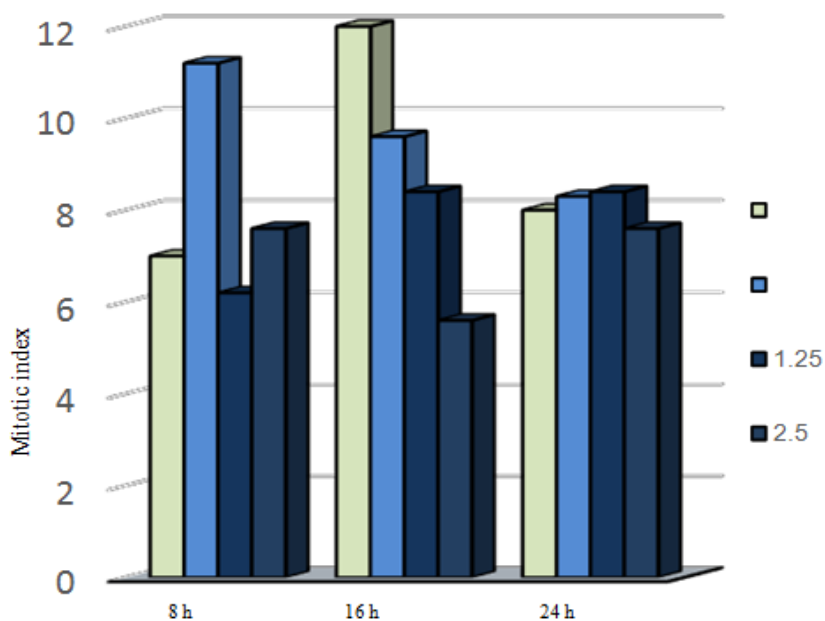
Tables 3 and 4 and Figures 9 and 10 show the types of scored chromosome anomalies, which were stickiness, C-metaphase, disturbance, anaphase bridge, micronuclei, lagging chromosome, disturbed polar, and bi-nucleic.

### Nucleic acid content

Table 5 and Figures 11 and 12 show 2-CP and 2,4-DCP effects on *A. cepa* DNA and RNA contents after treatment for 24 h. Treatments with 0.625 and 2.5% of 2-CP increased the DNA content (83.72, 58.64 ng/ $\mu$ l), respectively, compared with the control (55.5 ng/ $\mu$ l), and it was significant at  $p < 0.05$ , while treatment with 1.25% decreased the DNA content (32.92 ng/ $\mu$ l), and it was significant at  $p < 0.05$ . Also, treatment with 0.625 and 2.5% increased the RNA content (18.2, 14.7 ng/ $\mu$ l), respectively, compared with the control 10.1 ng/ $\mu$ l, while treatment with 1.5% decreased the RNA content to 7.1 ng/ $\mu$ l, and these effects were significant at  $p < 0.05$ .

2,4-DCP concentrations increased the DNA content after treatment for 24 h (72.44, 61.96, and 56.44 ng/ $\mu$ l), respectively, compared with the control 55.5 ng/ $\mu$ l, and the effect was significant at  $p < 0.05$ . Different treatments significantly affected the RNA content; it increased (29.05, 76.6, and 80.45 ng/ $\mu$ l) compared with the control 10.1 ng/ $\mu$ l.

Table 6 and Figures 13 and 14 show the effect of different treatments of 2-CP and 2,4-DCP on DNA and RNA contents of *V. faba* root tip cells. The 2-CP tested concentrations increased the DNA content (6.625, 6, and



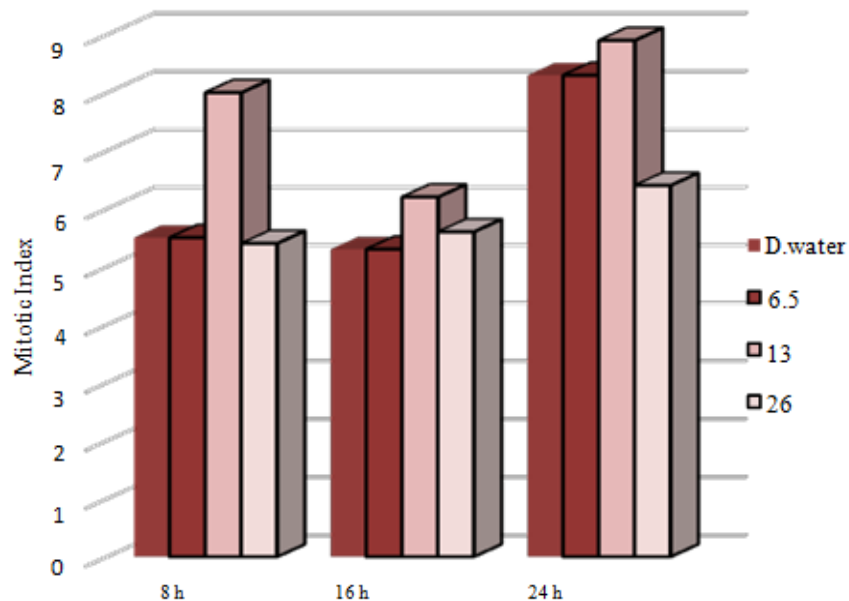
**Figure 1.** Effect of 2, chlorophenol on mitotic index of *Allium cepa* After treatment with different concentrations for different periods of time.  
Source: Author.

**Table 1.** Effects of different concentrations of chlorophenols for different periods of time on Mitotic index and chromosomal aberrations of *Allium cepa*.

Material	Concentration %	Time of duration/h	No. of total Cells	No. of normal cells	Mutant cells	MI (%)	CA
D. water	D- Water	8	2321	166	11	7	0.07
	D- Water	16	2034	244	13	12	0.05
	D- Water	24	2190	174	8	8	0.05
2-chlorophenol	0.625	8	2074	232	12	11.2	0.05
	0.625	16	2077	200	12	9.6	0.06
	0.625	24	2164	179	2	8.3	0.01
	1.25	8	2155	134	5	6.2	0.04
	1.25	16	2020	170	6	8.4	0.04
	1.25	24	2127	178	12	8.4	0.07
	2.5	8	2094	159	17	7.6	0.10
	2.5	16	2097	118	7	5.6	0.06
	2.5	24	2135	162	10	7.6	0.06
2,4-dichlorophenol	0.65	8	2152	118	8	5.5	0.07
	0.65	16	2246	119	9	5.3	0.08
	0.65	24	2219	184	31	8.3	0.10
	1.3	8	2237	177	9	8	0.05
	1.3	16	2567	159	14	6.2	0.09
	1.3	24	2661	238	22	8.9	0.09
	2.6	8	2045	111	10	5.4	0.09
	2.6	16	2350	132	15	5.6	0.11
	2.6	24	2686	172	14	6.4	0.08

Source: Author



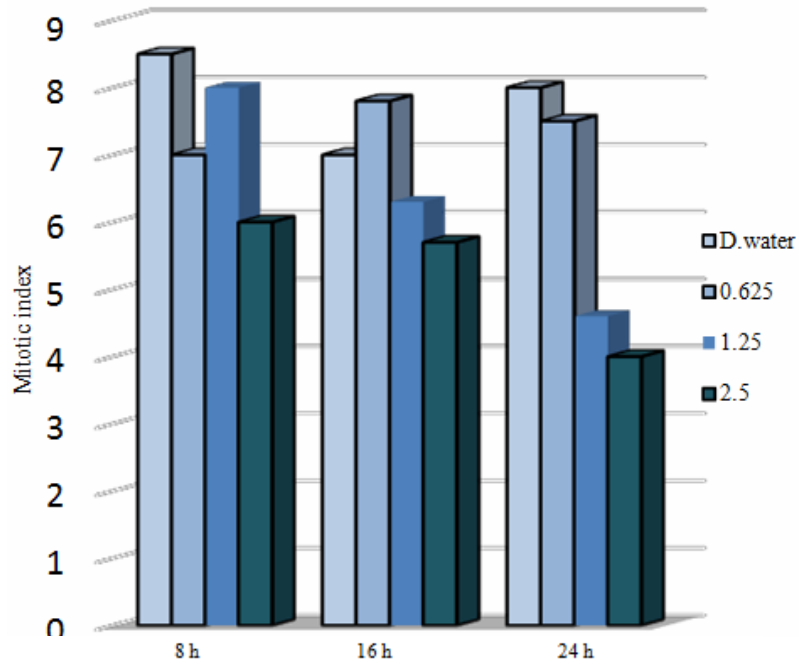


**Figure 2.** Effect of 2,4-Dichlorophenol on mitotic index of *Allium cepa* after treatment with different concentrations for different periods of time. Source: Author.

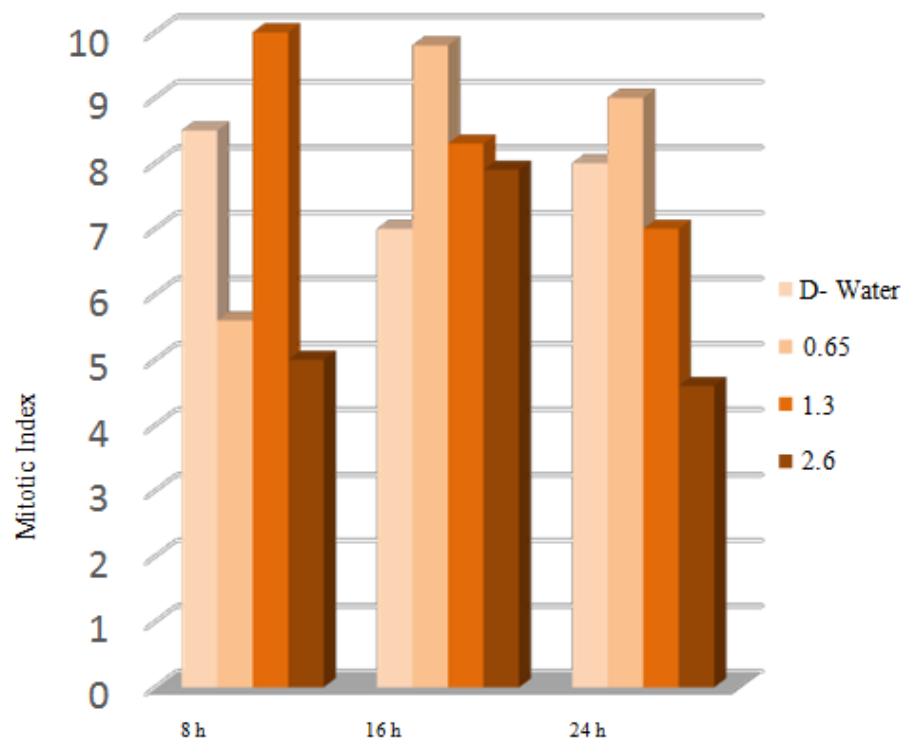
**Table 2.** Effects of different concentrations of chlorophenols for different periods of time on mitotic index and chromosomal aberrations of *Vicia faba*.

Material	Concentration (%)	Time of duration/h	No. of total Cells	No. of normal cells	Mutant cells	MI (%)	CA
D-water	D-Water	8	2145	183	9	8.5	0.05
	D-Water	16	218	155	2	7	0.01
	D-Water	24	2008	153	5	8	0.03
2-chlorophenol	0.625	8	2257	163	21	7	0.13
	0.625	16	2257	176	16	7.8	0.09
	0.625	24	2103	157	12	7.5	0.08
	1.25	8	2191	181	9	8	0.05
	1.25	16	2031	128	17	6.3	0.10
	1.25	24	2144	99	10	4.6	0.10
	2.5	8	2069	117	2	6	0.02
	2.5	16	2083	118	8	5.7	0.07
	2.5	24	2103	85	8	4	0.09
2,4-dichlorophenol	0.65	8	2183	123	24	5.6	0.20
	0.65	16	2206	224	17	9.8	0.08
	0.65	24	2050	186	17	9	0.10
	1.3	8	2052	205	31	10	0.20
	1.3	16	2235	186	28	8.3	0.20
	1.3	24	2022	139	13	7	0.10
	2.6	8	2048	109	15	5	0.10
	2.6	16	2071	164	25	7.9	0.15
	2.6	24	2133	98	21	4.6	0.20

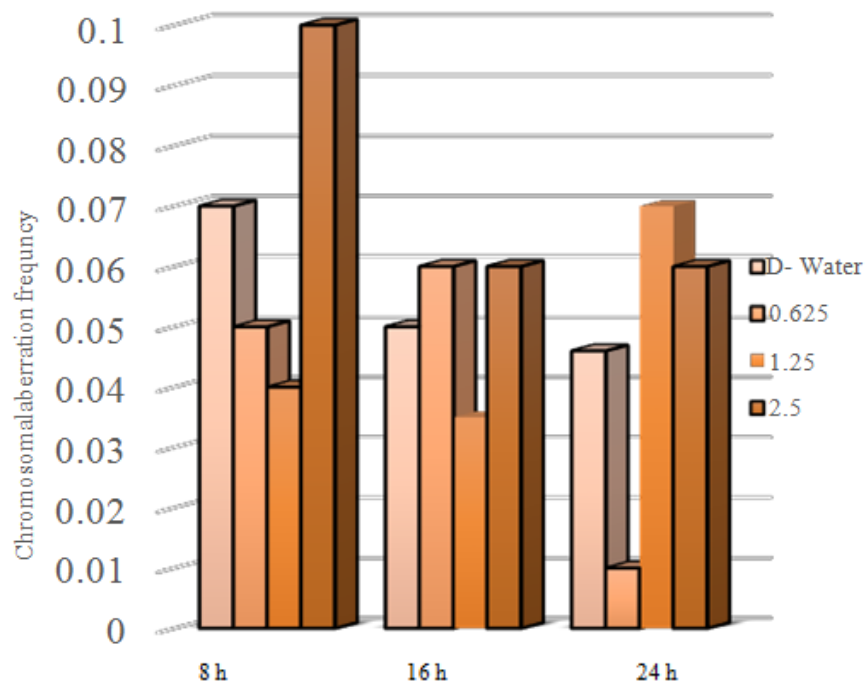
Source: Author



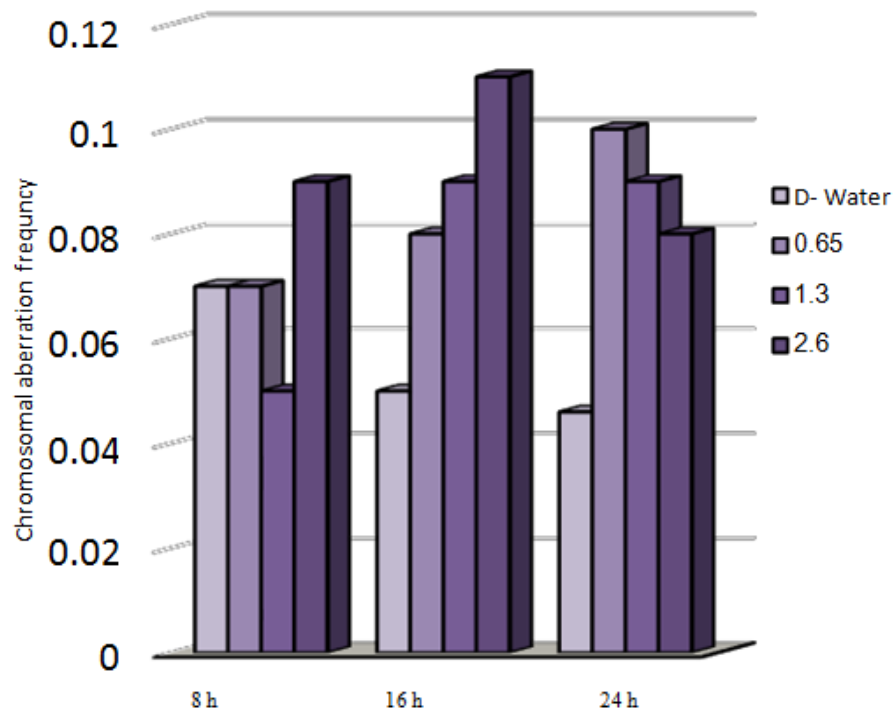
**Figure 3.** The effect of different concentrations of 2-chlorophenol for different periods of time on mitotic index of *Vicia faba* root tip cells. Source: Author.



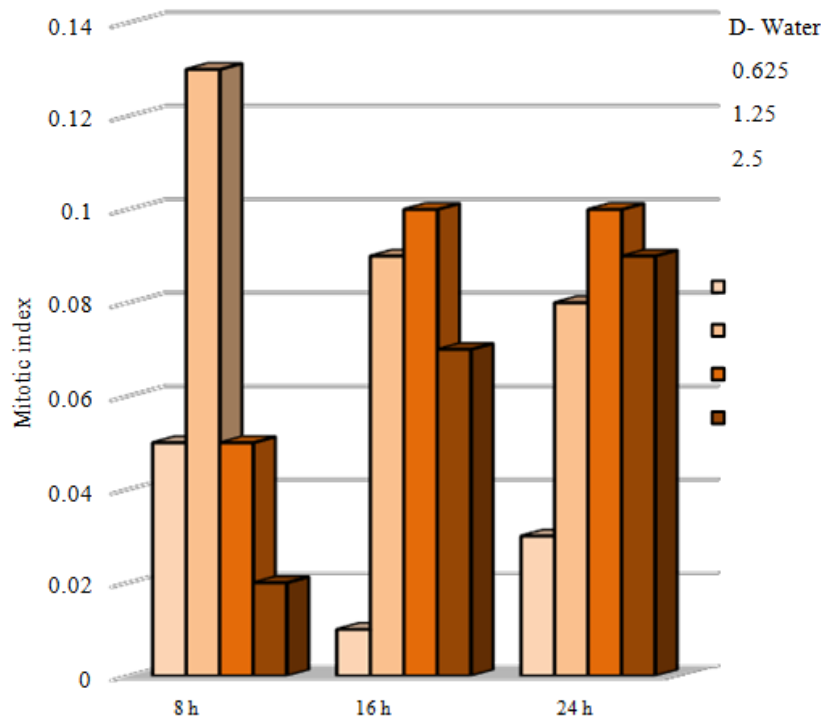
**Figure 4.** Effect of different concentrations of 2,4-dichlorophenol for different periods of time on Mitotic index of *Vicia faba* root tip cells. Source: Author.



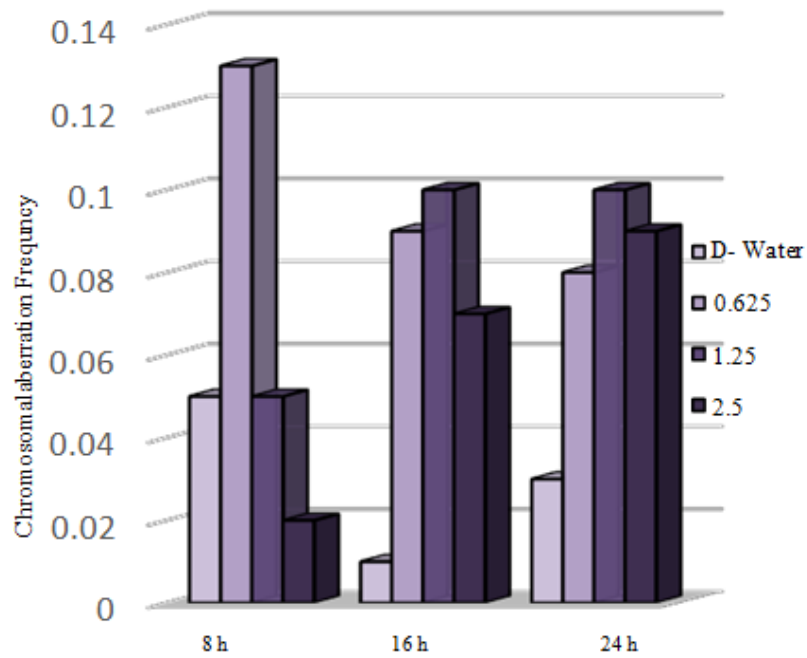
**Figure 5.** The effect of different concentrations of 2-chlorophenol for different periods of time on chromosomal aberrations frequency of *Allium cepa*. Source: Author.



**Figure 6.** Effect of different concentrations of 2,4-dichlorophenol for different periods of time on chromosomal aberrations frequency of *Allium cepa*. Source: Author.



**Figure 7.** Effect of different concentrations of 2-chlorophenol for different periods of time on chromosomal aberration frequency on root tip cells of *Vicia faba*. Source: Author.



**Figure 8.** The effect of different concentrations of 2,4-Dichlorophenol for different periods of time on chromosomal aberration frequency of *Vicia faba*. Source: Author.

**Table 3.** Types of chromosomal aberrations after treatment with different concentrations of 2, chlorophenol and 2-4, dichlorophenol for different periods of time on cells of root tip of *Allium cepa*.

Tested materials	Control	2-chlorophenol									2,4-dichlorophenol													
		0.625			1.25			2.5			0.65			1.3			2.6							
Type of CA	Cell phases	8	16	24	8	16	24	8	16	24	8	16	24	8	16	24	8	16	24	8	16	24		
Sticky	Metaphase	0.02		0.011																				
Disturb		0.04	0.006		0.02	0.02	0.005				0.02	0.009	0.008	0.006										
Lagging														0.006										
c-metaphase					0.004				0.007								0.006			0.007	0.02			
Fragment	Anaphase																							
s- metaphase																								
Sticky																								0.02
Disturb		0.006			0.004				0.007			0.02		0.008			0.006		0.004					0.008
C. anaphase	Telophase													0.008										
Lagging											0.02			0.02										
Bridge		0.006		0.03	0.02	0.04	0.005		0.02	0.03	0.02	0.03	0.02	0.008	0.02	0.02	0.04	0.004					0.04	0.02
Sticky									0.02															
Bridge	CA		0.006			0.005																		
Chromatin bridge														0.008										
dis polar																								0.006
bi-nucleus																0.005							0.004	
Micronuclei																	0.006							0.006
CA		0.07	0.01	0.05	0.05	0.06	0.01	0.04	0.04	0.07	0.02	0.06	0.06	0.07	0.08	0.2	0.05	0.09	0.09	0.14	0.11			0.08

Source: Author

4.76 ng/μl), respectively, and they were significant at p < 0.05; also, treatment with 2,4-DCP increased the DNA content (5.95, 22.15, 9.8 ng/μl) compared with the control 2 ng/μl, and it was significant at p < 0.05.

RNA contents were decreased by the tested concentrations of 2-CP after 24-h exposure (21.88, 24.88, and 21.84 ng/μl), respectively, compared with the control 25 ng/μl. Treatment with 0.65% and 2,4-DCP reduced RNA content (16.68 and 17.6 ng/μl), respectively, while treatment with 1.3% increased the RNA content

(28.6 ng/μl) compared with the control 10.1 ng/μl, and this effect was significant at p < 0.05.

### DISCUSSION

#### Mitotic Index (MI) and Chromosomal Aberration Frequency (CAF)

The mitotic index, MI (percentage of cells in mitosis at any time) offers a measure of the capacity of cells to divide and of the rate of cell

division (Campbell, 1983). The result showed that treatment with different concentrations of 2-CP and 2,4-DCP for different periods decreased the MI and increased the CAF compared with the control. This decrease in MI may be due to the MI depressive action of the tested materials interfering in the regular cell cycle (Chandra et al., 2002; INCEER et al., 2003; Sharma and Vig, 2012; Liman et al., 2015; Hoseiny-Rad and Aivazi, 2020). Hidalgo et al. (1985) suggested that DNA polymerase and other enzymes are among the tested material targets that induced

**Table 4.** Type of chromosomal aberration after treatment with different concentrations of 2-chlorophenol and 2,4-dichlorophenol for different period of time on cells of root tip of *Vicia faba*.

Tested materials	Control	2-chlorophenol									2,4, dichlorophenol											
		0.625			1.25			2.5			0.65			1.3			2.6					
Type of CA	Cell phases	8	16	24	8	16	24	8	16	24	8	16	24	8	16	24	8	16	24	8	16	24
Stiky		0.02			0.02	0.02	0.06	0.02	0.02	0.03	0.06	0.008	0.001	0.04	0.02	0.01	0.005	0.04	0.02	0.03	0.01	0.01
Distirb		0.02	0.006	0.01	0.03	0.05	0.01	0.01	0.07	0.05	0.03	0.02	0.02	0.07	0.03	0.05	0.04	0.03	0.07	0.07	0.09	0.1
Lagging	Metaphase							0.006								0.03						
c-metaphase					0.02						0.03	0.008	0.02	0.03			0.01	0.04		0.006	0.04	
s- metaphase						0.006				0.01							0.02				0.01	
Stiky		0.005						0.006			0.01	0.008			0.004							
Distirb	anaphase			0.01	0.03				0.008		0.06	0.03		0.02	0.004	0.005		0.005	0.02			0.01
Lagging						0.006			0.008		0.01			0.008		0.005		0.005				
Bridg		0.005		0.01	0.02	0.01		0.006	0.008	0.01		0.008	0.02	0.02	0.02	0.02	0.005	0.03	0.02	0.02	0.01	
Sticky											0.01											
Distirb											0.01											
Lagging	telophase										0.03						0.005					0.006
Bridge			0.006						0.008							0.02						0.01
Fragment																					0.009	
bi-nucleus					0.006			0.006	0.02	0.01										0.02	0.01	0.04
Micronucli												0.01								0.009		
CA		0.05	0.01	0.03	0.13	0.09	0.08	0.05	0.1	0.1	0.3	0.07	0.09	0.2	0.08	0.1	0.2	0.2	0.2	0.15	0.2	0.2

Source: Author

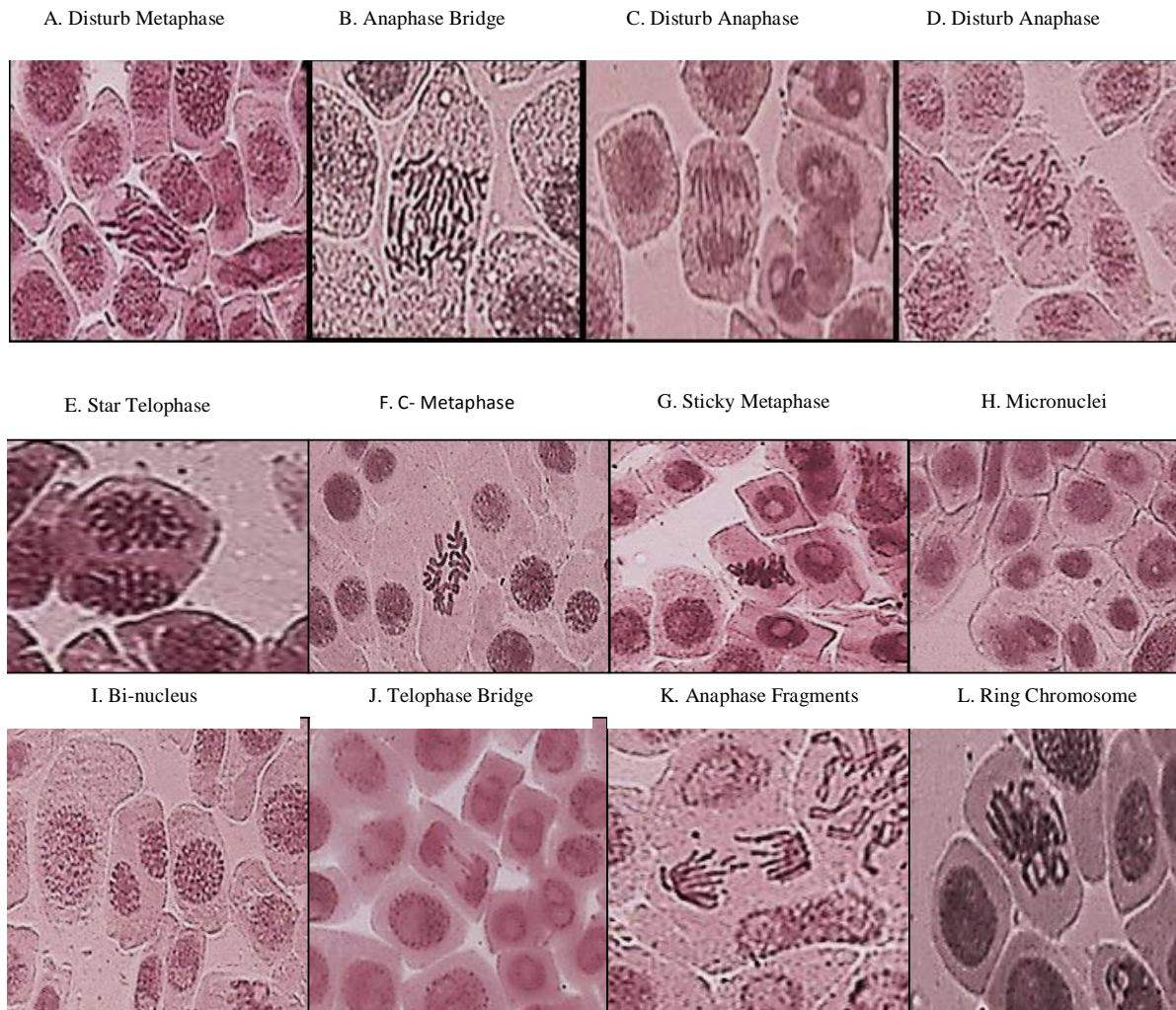
antimitotic effects. Lehnen et al. (1990) found that MI decrease is attributed to the inhibition of DNA synthesis and formation of irregular and disorganized phragmoplasts. Öcal and Eroğlu (2012) suggested that the MI decrease may be due to the possible cellular death or delay in cell proliferation. Also, Hansch et al. (2000) concluded that phenol toxicity is related to free radical formation. A similar result was found by Dimitris et al. 2016, Kumar et al. (2010), Lone et al. (2013), and Kumar and Srivastava (2015).

Chromosomal aberration assay for cytological

aberrations is a useful and sensitive test for detecting genotoxins (Sheila, 2000).

The results revealed that treatment with 2-CP and 2,4-DCP caused several types of chromosomal aberrations, and the most noticed chromosome abnormalities that appeared after treatment with 2-CP were stickiness, disturbed, and anaphase bridge; other types of abnormalities but in low percentage were lagging chromosomes, fragment, C- metaphase, S-anaphase, bi-nucleus, micronuclei, and irregular poles. 2,4-DCP strongly affected

spindle function, causing the appearance of disturbance, C-metaphase, S-anaphase, and laggards. This effect was caused by binding to a protein subjected to spindle microtubule (Brinkley et al., 1985; Pavlica et al., 1991). Chromosome stickiness can be due to the effect of the chromosomal proteins or disturbances in the functioning of specific non-histone proteins essential for chromatid separation and segregation (Gaulden, 1987); also, chromosome stickiness may occur because of chromosomal protein, which is attributed to the irregular



**Figure 9.** Types of chromosomal anomalies after treatment with different concentrations of tested substance for different periods of time in root tip cells of *Allium cepa*.  
Source: Author

folding of chromosome fibers or the action on the polymerization process (El-Ghamery et al., 2000). Mahakhode and Somkuwar (2013) suggested that it may occur due to the sudden contraction of some spindle fibers. Kumar and Srivasatava (2015) reported that scattering (disturbance) and spindle dysfunction were attributed to the loss of the spindle fibers, microtubules.

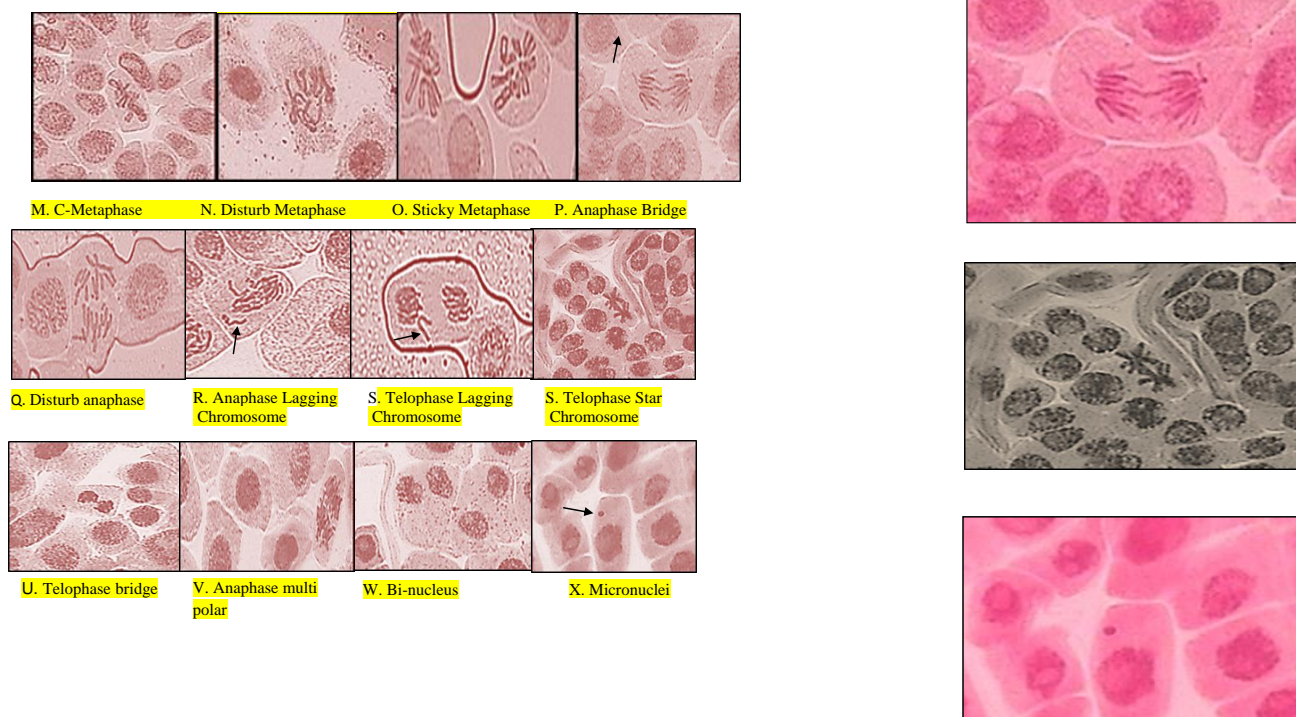
C-mitosis during metaphase and anaphase were also scored, and this type of abnormality can be due to the effect of the tested material on spindle fibers. Morejohn and Fosket (1986) proposed that C-mitosis appears because of mitosis blocking at metaphase and depolymerizes spindle microtubules; a similar result was found by Dimitrov and Gadeva (2006).

Chromosome fragments appeared because of chromosome breaks (chromatids). Kumar and Singh (2004) reported that chromatids' breakage had been

linked to DNA synthesis, which is sensitive to many chemicals. Kumar et al. (2010) concluded a comparable result.

The chromosome bridge was formed from the sticky behavior of chromosomes that cannot move toward pole regions at anaphase (Kumar and Rai, 2007), it may also be due to the chromosomal instability (Ganem et al., 2009); a similar result was found by Kumar and Srivastava (2015). Sinha (1989) suggested that chromosome bridge was attributed to the formation of dicentric chromosomes due to breakage and reunion.

For micronuclei definition and scoring, the micronuclei criteria by Tolbert et al. (1991) were followed. The failure of the movement of lagging chromosomes increases micronuclei (Kumar and Dubey, 1998). Dichlorophenol is active in the G<sub>1</sub> and S phases of the cell cycle (Arzt et al., 1989; Lone et al., 2013).



**Figure 10.** Types of chromosomal anomalies after treatment with different concentration of tested subsentence for different periods of time in root tip cells of *Vicia faba*.  
Source: Author

**Table 5.** DNA and RNA Concentration of *Allium cepa* after treatment with different concentrations of 2, CP and 2-4, CP for 24 h.

Tested materials	2, CP				2-4, DCP		
	D. water	0.625	1.25	2.5	0.65	1.3	2.6
DNA (ng/μl)	55.5	83.72	32.92	58.64	72.44	61.96	65.44
RNA (ng/μl)	10.1	18.2	7.1	14.7	29.05	76.6	80.45

Source: Author

**Table 6.** DNA and RNA Concentration of *Vicia faba* after treatment with different concentrations of 2-CP and 2,4-CP for 24 h.

Tested Materials	2-CP				2,4-DCP		
	D. water	0.625	1.25	2.5	0.65	1.3	2.6
DNA. ng/μl	2	6.25	6	4.76	5.95	22.15	9.8
RNA. ng/μl	25.56	21.88	24.88	21.84	16.68	28.6	17.6

Source: Author

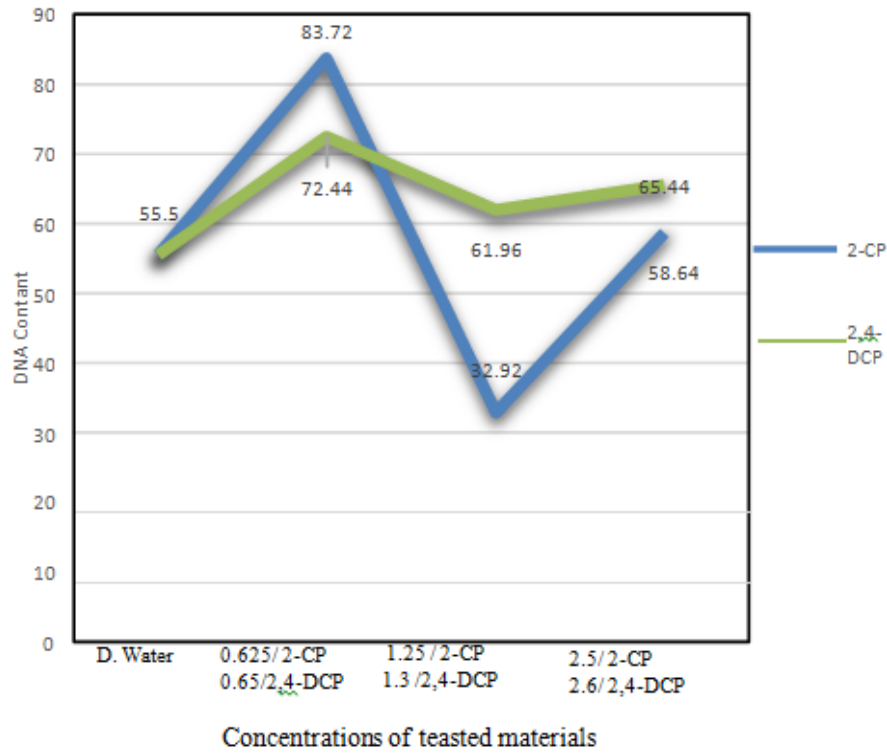
**Nucleic acid content**

The nucleic acid biosensor can be used to detect chemical species (Palchetti and Mascni, 2008). Treatment with different concentrations of 2-CP for 24 h affected the DNA content compared with the control. The lowest and highest concentrations of 2-CP increased the DNA

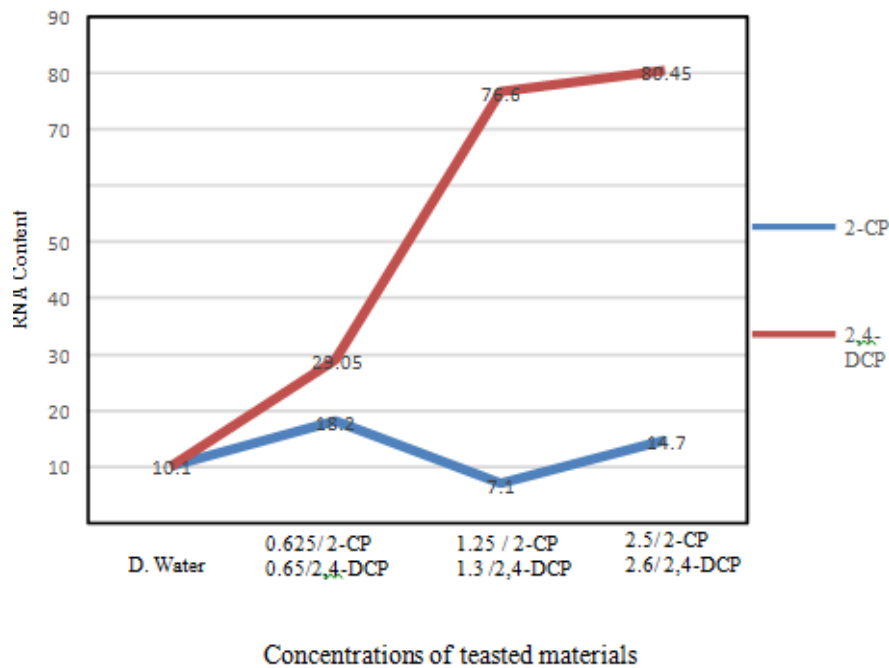
content of *A. cepa*, and the half concentration decreased it, while treatment with 2,4-DCP concentrations increased the content of DNA. RNA was also affected by DNA concentration; RNA content was directly proportional to DNA content.

In *V. faba* treatments with different concentrations of 2-CP and 2,4-DCP, DNA content was increased;

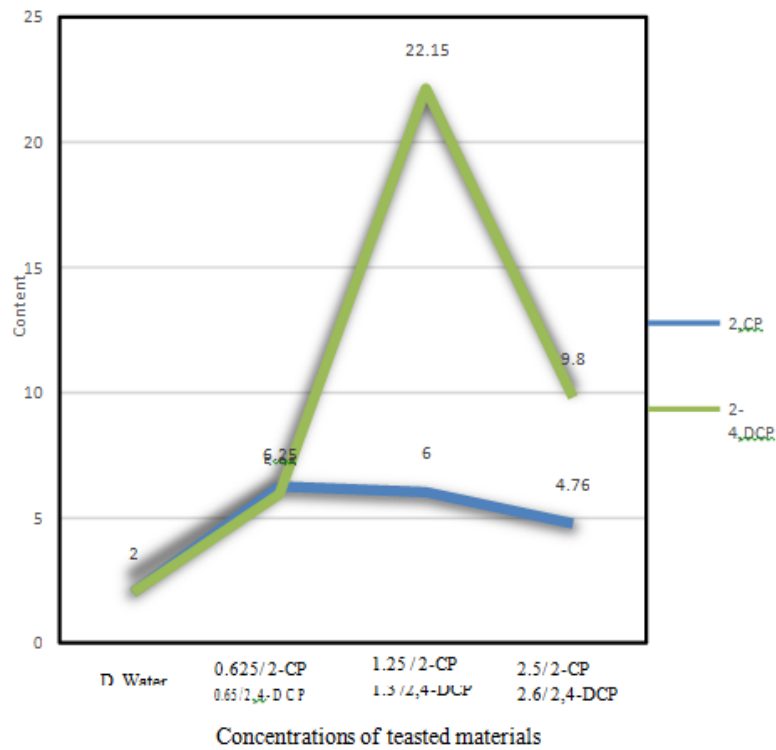




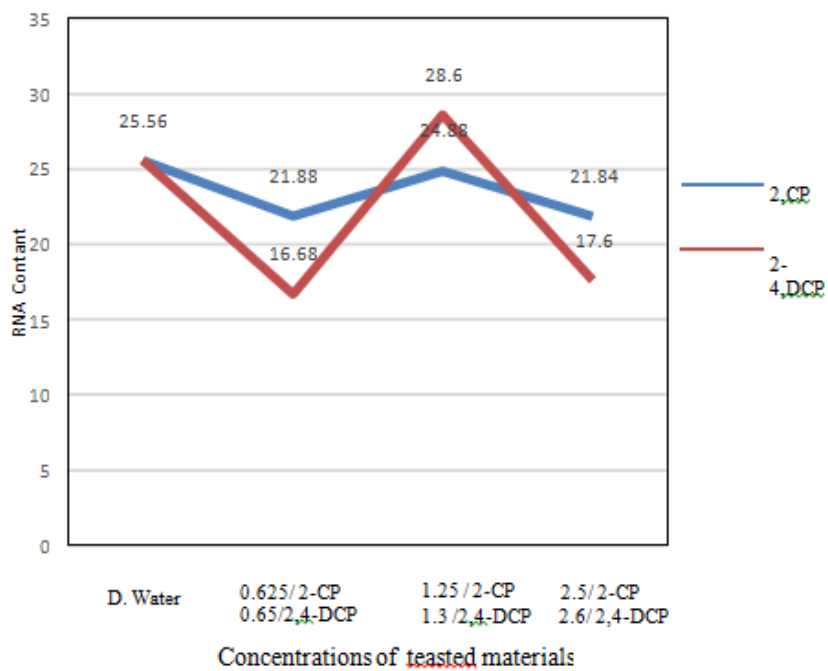
**Figure 11.** Effect of different concentrations of 2, CP and 2- 4,DCP on *Allium cepa* DNA content.  
Source: Author.



**Figure 12.** Effect of different concentrations of 2,CP and 2-4,CP on *Allium cepa* RNA content.  
Source: Author.



**Figure 13.** Effect of different concentrations of 2,CP and 2- 4,DCP on *Vicia faba* DNA content.  
Source: Author.



**Figure 14.** Effect of different concentrations of 2,CP and 2- 4,DCP on *Vicia faba* RNA content.  
Source: Author.

treatments with different concentrations of 2-CP decreased the RNA content compared with the control. While treatment with half concentration of 2,4-DCP increased it.

The increased DNA content may be due to the low concentration dosage. El-Hadary and Gyuhwa (2013) suggested that herbicides in low doses act as growth regulators, while a decrease in DNA may be due to the effect of dosage on cells biochemical activities. Sharma et al. (2017) concluded that pesticides cause oxidative stress in plants by producing reactive oxygen species (ROS). Plants resist pesticide toxicity by activating the internal antioxidation defense system, which includes an antioxidative enzyme and non-enzymatic antioxidation. A similar result was found by Kuckl and iman (2018) when *A. cepa* was treated with 2-CP; it may also be due to the action of the tested concentrations, which was insufficient to counteract the oxidative damage induced by the overproduction of ROS (Fernades et al., 2020). Duchnowicz et al. (2002) explained that the toxicity of higher concentrations of 2,4-DCP decreases ATPase activity. 2,4-DCP induced ROS overproduction and DSBs, showing that ROS accumulation and GSH depletion are involved in 2,4-DCP, causing DNA damage in fish (Huang et al., 2018). A similar result was found by Tingting et al. (2017) in membrane, which induced a change in ion transport and disruption of both sides of the membrane, agreeing with the result of Bukowska (2006; Michalowice and Majsterek 2010).

## Conclusion

The tested materials (2-CP and 2,4-DCP) showed that different concentrations affected the MI and CAF in both plants (*A. cepa* and *V. faba*). Several types of chromosome anomalies were scored, such as stickiness, disturbance, anaphase bridges, micronuclei, and binuclei. Although nucleic acid contents (DNA and RNA) increased after treatment with different concentrations (0.625 and 2.5%) of 2-CP and (0.65, 1.3, and 2.6%) of 2,4-DCP, they decreased by 1.25% of 2-CP. The increase in DNA and RNA content may be due to protective action by protein production that allows the plant to continue the biological process normally under chemical stress, or it may be due to the interference of chemicals with the cell cycle by disturbing the normal flow of the process, leading to DNA content accumulation and decreasing cell division MI. The tested materials have cytogenetic, genotoxic, and also influence in nucleic acid contents in *A. cepa* and *V. faba* plants.

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## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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*Full Length Research Paper*

# Characterization of plant growth promoting rhizobacteria isolated from soils of Senegalese Semi-arid Sahelian Zone

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The semi-arid Sahelian region of Africa, characterized by its deleterious agro-climatic conditions, is also subject to agricultural overexploitation to support human and livestock food. This situation has strongly accentuated the continuous degradation of arable land. However, green biotechnologies are often proposed to promote more sustainable agricultural systems. Thus, the soil microbiome is explored for the production of biofertilizers that are able to ensure the growth and biocontrol of cultivated plants. For this purpose, plant growth promoting rhizobacteria (PGPR) were widely used. In order to increase the production of fodder for livestock, the production of biofertilizers is undertaken using indigenous PGPR strains able to sustainably improve agricultural productivity. From soil samples collected in the investigated zone, rhizobacteria are isolated and characterized *in vitro* for a selection of elite strains. Thus, a collection of 71 isolates presenting a great phenotypic and physiological diversity was established. The most efficient strains were selected for their ability to promote plant growth by the production of catalase and siderophores, auxin synthesis and soil P bioavailability. At the end of this study, candidate strains for the formulation of a suitable biofertilizer were highlighted on the basis of the correlations between the highest PGP activities of bacterial strains.

**Key words:** Biofertilizers, plant growth promoting (PGP) traits, Rhizobacteria, Sahelian zone.

## INTRODUCTION

Sahelian semi-arid environment in Africa is generally characterized by low rainfall and poor soils nutrient. This constitutes a challenge for crops to thrive on such soils due to environmental degradation. Furthermore, in

Senegal, livestock farming is developed in the agro-ecological areas subject to these very devastating geo-climatic conditions. However, in recent years, Senegalese breeding has been marked by an increase in herd

numbers which has naturally led to an increase in fodder requirements. Indeed, the rapid expansion of cultivated areas, recurrent droughts, erosion and multifaceted degradation of soils due to human and animal actions have greatly reduced grazing areas (Ickowicz and Mbaye, 2001). The synergic action of these factors has resulted in increasing difficulties in feeding livestock.

In this context of fodder deficit added to hostile agro-climatic conditions in this region, it would be necessary to exploit the potential of adapted fodder plants or at least double-purpose plants (grain and fodder) such as fodder millet and leguminous crops like peanuts and cowpeas which could be good models. However, it has been shown that microbial activity in soil is intense, particularly under the influence of the roots due to the high presence of microorganisms in the rhizosphere (Canbolat et al., 2006). Some of these beneficial soil microorganisms interact with plants and considerably help plants for the improvement of nutrition and development by their functional diversity (Chitraselvi et al., 2015). These microorganisms draw in soils the nutrients released by the plant roots, used as energy sources necessary for their metabolism (Bouras, 2018). Thereby, they play an important role in plant growth and development (Afzal and Bano, 2008). They perform also their activities due to their interaction with soil characteristics and nutrient availability (Yazdani et al., 2009).

The heterogeneous group of bacteria usually found in the rhizospheric soils of plants, which can improve the extent or quality of plant growth directly and/or indirectly are qualified as PGPR (Vessey, 2003; Bouali, 2017). The PGPR produce or modify the concentration of plant growth regulators like indole acetic acid (IAA), gibberellic acid, cytokines and ethylene (Backer et al., 2018). Others beneficial effects of these microorganisms to crops are the asymbiotic binding of  $N_2$  (Ahemad and Kibret, 2014), antagonism against phytopathogenic microorganisms by production of siderophores (Kaioua and Grairi, 2015), antibiotics (Backer et al., 2018), 1-amino-cyclopropane-1-carboxylate (ACC) deaminase, and hydrogen cyanate (Liu et al., 2016) and cyanide (Sehrawat et al., 2022). Some bacteria also have the ability to solubilize phosphate minerals and others nutrients, promote the circulation of nutrients and minimize the need for chemical fertilizers (Canbolat et al., 2006; Gouda et al., 2018). For this, there is growing interest in increasing the contribution of PGPR to the growth of crop plants in agriculture. The application of bacteria that promote plant growth can play an important role in the production of organic fodder and contribute to reduce environmental degradation (de-Bashan et al., 2012). Taking account to all these bacterial benefices to plants, preliminary study was undertaken with the objective to detect potential

PGPR in the soil of the agro-sylvo-pastoral area for fodder plant biofertilizing, growth-stimulating and biocontrol. Thereby, the best performing will be screened and selected for the formulation of bio-fertilizer to substitute synthetic fertilizers in order to overcome the problems of declining fertility and environmental pollution, while improving crop productivity.

## MATERIALS AND METHODS

### Isolation of soil bacteria

Soil samples were collected from the 0 - 20 cm topsoil of plots with previous crops of cereals and leguminous in the agrosylvo-pastoral area of Dahra (latitude 51°21'N, longitude 15°29'W) in the Senegalese semi-arid Sahelian zone. Soils samples were mixed to form a composite sample and then air dried at 22°C. To isolate rhizobacterial strains, 10 g of ground soil were added to flask containing sterilized 50 mL of phosphate-buffered saline (PBS). This mixture was stirred for 2 h. The soil suspension obtained was shaken for 2 h at 28°C.

A tenfold dilution series to  $10^{-3}$  was performed by mixing 100  $\mu$ L of the soil suspension with 900  $\mu$ L of PBS in a tube. The culture was carried out by spreading 50 to 100  $\mu$ L of each dilution in Petri dishes containing Luria-Bertani (LB) agar medium. For each dilution, 3 repetitions were considered. The Petri dishes were incubated in the oven at 28°C for 24 h. Twenty-four hours after incubation, bacterial colonies grown on the medium were purified. Isolates were then selected on the basis of the following morphological characteristics: size, shape, colour, opacity and surface of the colonies.

### Characterization of rhizobacterial isolates

All isolates were grown individually into 10 mL of LB medium at 30°C. At exponential growth phase, series of analysis were performed for Gram bacteria, catalase reaction, auxin production, P solubilization, siderophore and pyoverdinin productions.

### Gram test of bacteria

Each bacterial isolate was subject to Gram test by using KOH solution. A drop of 3% KOH solution is brought into contact with a bacterial suspension on a slide by performing a circular movement with a sterile toothpick. The viscosity of KOH solution (presence of filament) indicated Gram-negative response of bacteria. The reaction was considered positive when the viscosity appeared after 30 s (Dash and Payyappilli, 2016).

### Catalase activity

Separately cultivated isolates were all tested for catalase activity as indicated by Martins and English (2014). In this way, a drop of  $H_2O_2$  was placed on slides containing 10  $\mu$ L of the bacterial suspensions. Positive catalase activity was revealed by the amount of gas released as bubbles (effervescence) reflecting the decomposition of oxygen.

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### Determination of IAA production

The auxin production capacity of the bacterial strains was tested according to the technique described by Siddhi et al. (2014). A 25  $\mu$ L volume of bacterial suspension was transferred to 4 mL of LB medium supplemented with 1 g tryptophan. After the incubation period (48 h) at room temperature, 2 mL of the liquid culture was centrifuged at 8000 rpm for 10 min. A volume of 0.5 mL of the supernatant was mixed with 1 mL of Salkowski's reagent and exposed to darkness for one hour. For each isolate, three replicates were performed. Auxin production was revealed by the development of a pink colour. The intensity of coloration was measured by spectrophotometer (530 nm). Estimation of auxin production was made by comparison to a standard curve established from an IAA solution.

### Estimation of phosphate solubilisation

The ability of the bacterial strain to solubilize inorganic phosphate was tested on solid Pikovskaya's medium (PVK) containing  $\text{Ca}_3(\text{PO}_4)_2$  as the sole source of P (Pikovskaya, 1948). The occurrence of a halo around the colonies indicates the production of phosphorus-dissolving substances (Desai and Amaresan, 2022). For each strain, aliquots (5  $\mu$ L) of bacterial cultures were spot inoculated on solid PVK medium at a rate of 3 replicates per isolate. After incubation of bacterial cultures at 28°C for 7 days, the P solubilization index (PSI) was calculated following using the formula proposed by Siddhi et al. (2014).

$$PSI = \frac{\text{colony diameter} + \text{halo zone diameter}}{\text{colony diameter}}$$

### Siderophores production

The detection of siderophores synthesis was carried out in the Chromium Azurol Sulfate (CAS) medium according to the modified method described by Pérez-Miranda et al. (2007). A volume of 5  $\mu$ L of a 24 h old culture of each isolate in liquid LB medium was spot deposited on the CAS medium as proposed by Patel et al. (2018) with 3 replicates per isolate. After 3 days of incubation at 28°C, the appearance of halo around bacterial colonies indicated siderophores production of considered isolates. The siderophores' production was evaluated using the siderophore index (SI) determined with the following formula:

$$SI = \frac{\text{colony diameter} + \text{halo diameter}}{\text{colony diameter}}$$

### Detection of pyoverdine production

The production of pyoverdine was investigated on solid King B medium (specific to *Pseudomonas*) to detect the isolates unable to fluoresce. Thus, 5  $\mu$ L of the bacterial suspension were inoculated on King B agar with 3 replicates. The dishes were then incubated at 28°C for 24 to 96 h. The appearance of a fluorescent yellow-green pigment was detected visually or under ultraviolet (UV) light at 365 nm (King et al., 1954).

### Statistical analysis

All data were subjected to analysis of variance (ANOVA) performed using XLSTAT (version 2010) software. Mean values of all treatments were compared using Newman-Keuls test (HSD) at the

significance level ( $p < 0.05$ ). The ascending hierarchical classification (AHC) method was used to group strains into clusters. Principal Component Analysis (PCA) was used to graphically represent the correlation between the PGP activities of the strains.

## RESULTS

### Isolation and phenotypic characterization of bacteria

On the basis of macroscopic characters of bacterial colonies on LB medium, 71 bacteria were isolated from Dahra soils. The dominant isolates were those which are regular in shape, white, opaque, creamy and with a smooth surface. Since their cultural and morphological characteristics were determined, all isolates were subject to biochemical test. For Gram test reaction, 74.65% of isolates were identified Gram-negative when the remained 17 isolates shown positive Gram reaction. On the basis of fluorescence emission detected by visual observation or under ultra-violet light, 21 isolates, representing 29.57% emitted a green fluorescence on the King B medium.

### Plant growth promoting characteristics of isolates

#### Catalase activity

The effervescence production was observed indicating a qualitative bacterial catalase activity. Thus, the results showed that 55 isolates corresponding to 77.46% have a positive reaction by catalysing hydrogen peroxide.

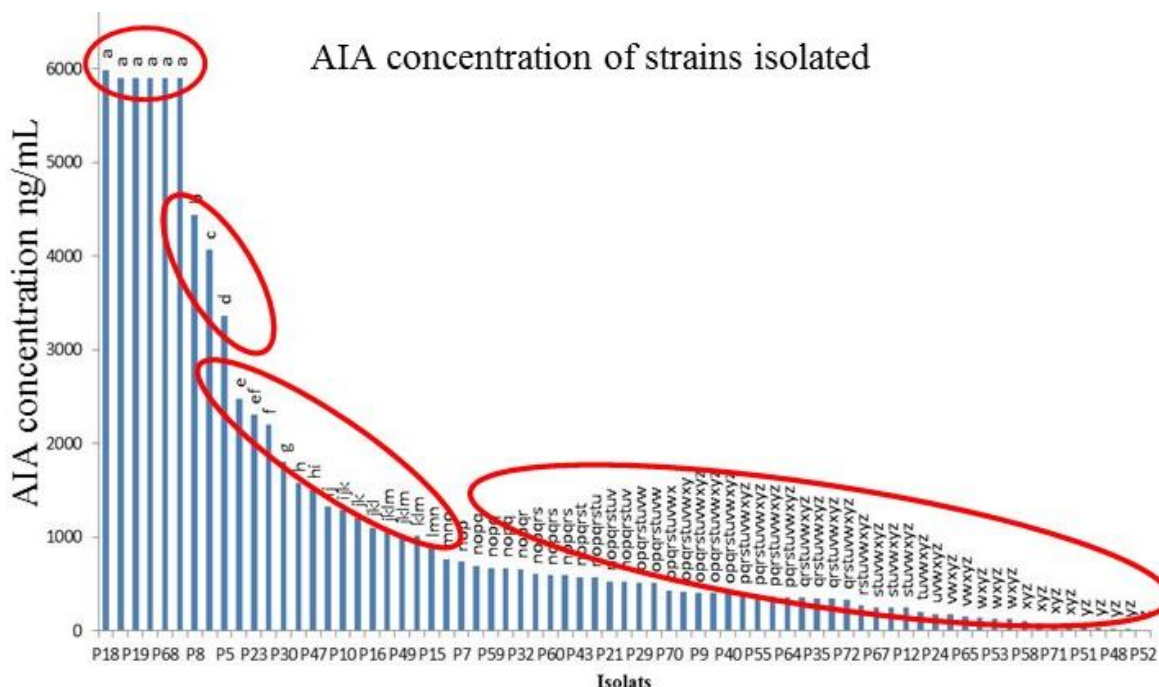
#### Auxin production (IAA)

The results showed that all isolates were able to synthesize auxin at variable degrees of intensity in the presence of tryptophan. The concentration of auxin produced varied significantly between isolates and was ranged from 12.1 to 5985.2 ng/mL.

The AHC analysis allowed the classification of the strains into 4 groups (Figure 1). A first group of 6 strains with concentration of IAA between 5985.2 and 5900.8 ng/mL. The second group with 3 strains whose concentration produced in IAA was between 4070.8 and 3367.2 ng/mL. In the third group of 14 strains, the concentration of IAA produced ranged from 2205.5 to 1575.2 ng/mL. For the last group of 48 strains, the concentration of IAA was between 741.2 and 329.5 ng/mL.

#### P solubilization of isolates

The solubilization potential of inorganic P of isolates was assessed by the value of the solubilization index (PSI)



**Figure 1.** Auxin production of isolated rhizobacterial strains. N.B. Due to the large numbers of isolates, some isolates are not shown on the x-axis of the figure. Source: Author

corresponding to the efficiency of an isolate in solubilizing P. Results showed after 7 days of incubation, that 80.28% of the bacterial isolates solubilized the phosphate with an index equal to 7.3.

Calculation of the PSI indicated that there was a significant difference in terms of P solubilization between the strains. Thus, the analysis of ascending hierarchical classification made it possible to classify our bacteria into 4 groups (Figure 2). The first group comprised a single isolate (P39) with the maximum value of solubilization index evaluated at 7.3. The last group of 14 strains does not solubilize P.

### Production of pyoverdine and siderophores

Fluorescence emission related to pyoverdine production was detected 4 days after incubation on the King B medium. The production of siderophores revealed by the presence of the orange halo analysed 7 days after incubation. It was noted that 29.65% of the strains were fluorescent when siderophores are produced by 47.89% of the isolated strains. The strains that did not produce siderophores were more representative in the analysed soil.

All isolates were classified into 3 groups (Figure 3) when considered the 37 bacterial isolates which did not produce siderophores. The first group consists of a single strain P64 with the maximum production value of 11.8. A

second group of 33 isolates whose production was ranged between 3.6 and 2.6. Among the two groups of siderophore-producing isolates, 21 strains fluoresce and produce pyoverdine.

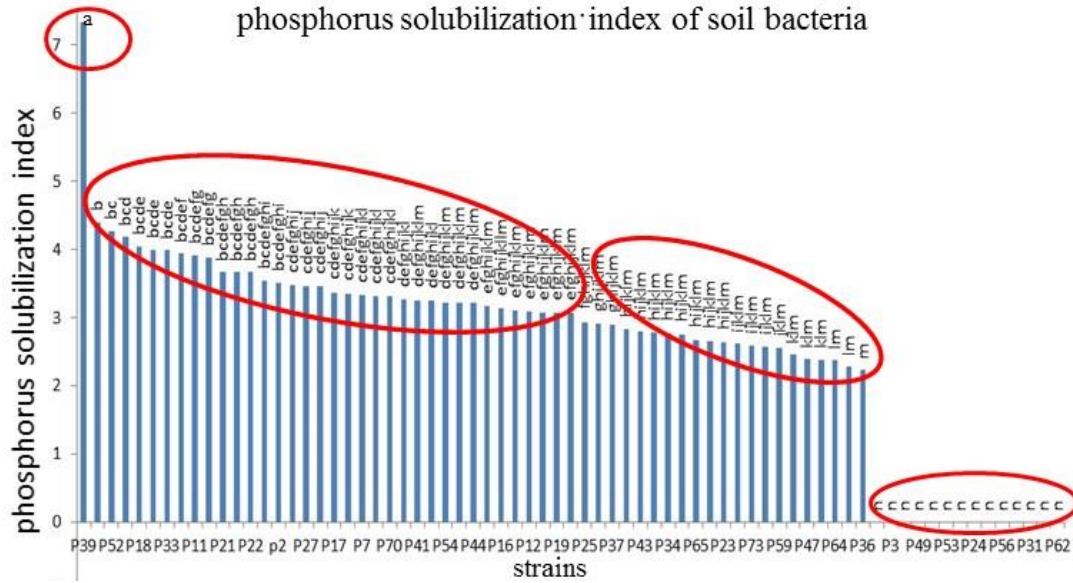
### Correlation matrix between the different variables and strains

Principal Component Analysis (PCA) was performed to assess the correlations between the PGP activities of bacterial strains (Figure 4). The 2 axes F1 and F2 (theoretical variables) from which the 2 dimensional graphs were obtained, represented 44.46 and 31.79% of the variability. They allow the maximum number of results to be represented in the table (76.25% of the variance is described). This percentage is largely sufficient for a PCA with significant interpretation.

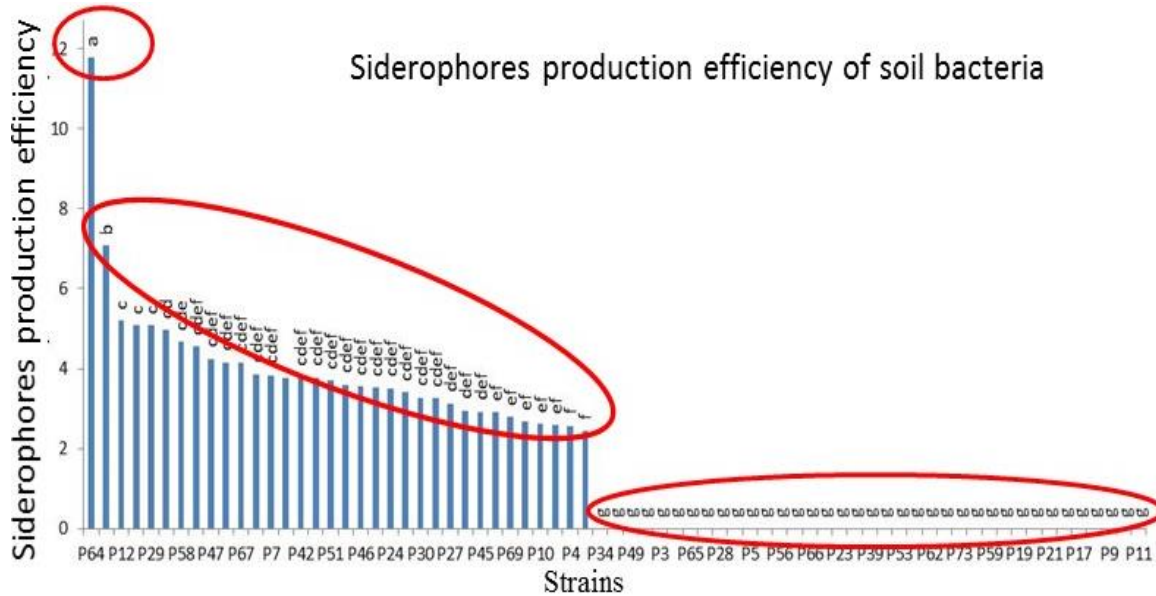
According to the results of the PCA, the production of auxin is negatively correlated with the P solubilization index. On the other hand, a strain which produces siderophores tends not to synthesize auxin. The correlation is positive between SI and siderophores production.

Strains P1, P18, P8, P5, P11, P23, P40, P39, P34 and P64 are highly auxin-producing. Likewise, strains P51, P38, P44, P36, P12, P15 and P58 produce siderophores at interesting rates. The strains which solubilize more P are P4, P22, P2, P22, P28 and P39. Strain P39 is a good





**Figure 2.** Phosphorus solubilization index of isolated rhizobacterial strains. N.B. Due to the large numbers of isolates, some isolates are not shown on the x-axis of the figure. Source: Author

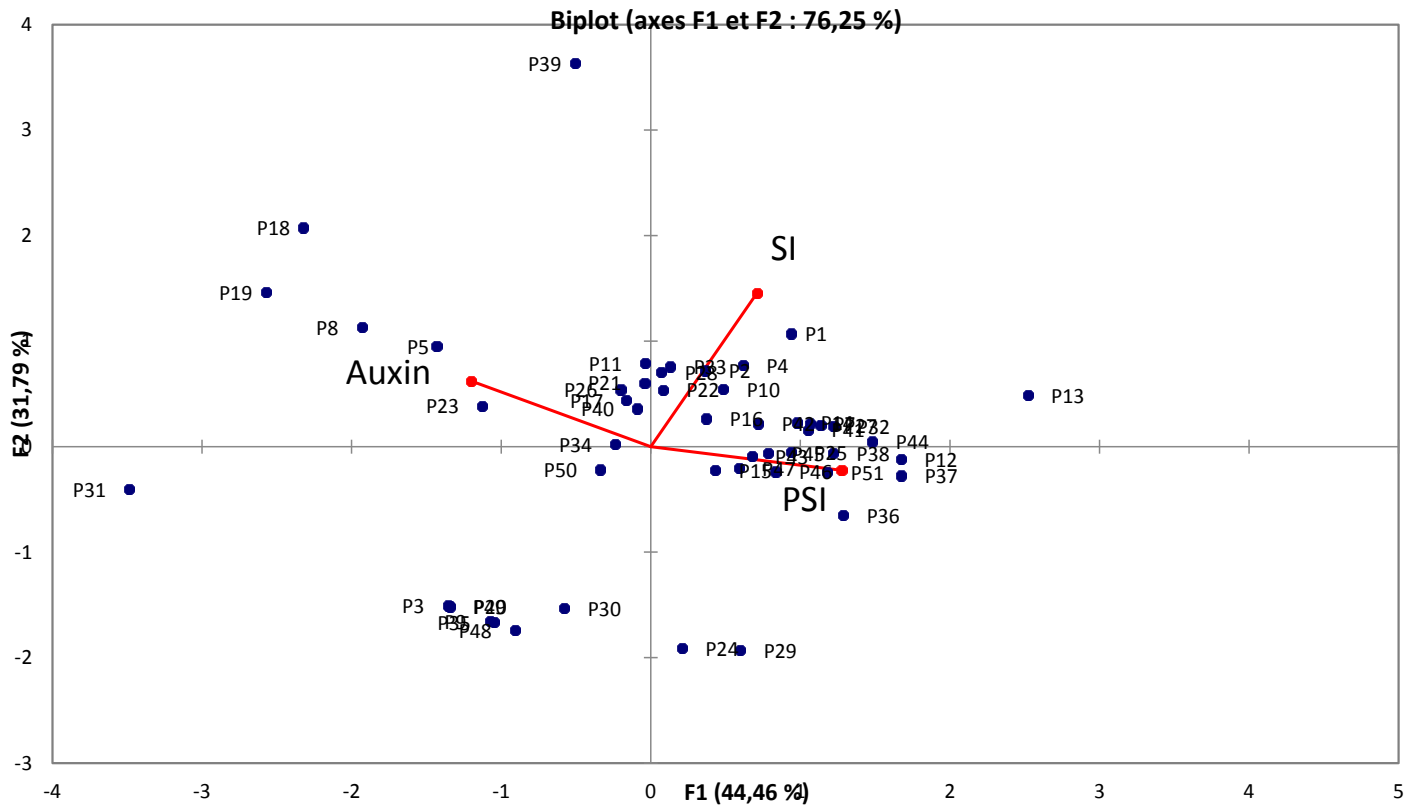


**Figure 3.** Siderophores production efficiency of rhizobacterial strains. N.B. Due to the large numbers of isolates, some isolates are not shown on the x-axis of the figure. Source: Author

candidate because of its character to produce interesting amounts of auxin and solubilize of large amounts of P. Strains P1, P64 and P58 synthesize auxin and siderophores and solubilize P. Strain P51 is a good candidate for the production of siderophores and also for the solubilization of P.

**DISCUSSION**

PGPR are beneficial rhizospheric bacteria that enhance plant health and growth (Vesey, 2003; Amar et al., 2013; Das et al., 2020). They are reported to be widely represented in soil and presented various activities for



**Figure 4.** Principal component analysis of PGP activities and rhizobacterial strains. Auxin: Auxin production; SI: Siderophore index; PSI : phosphate solubilization index.

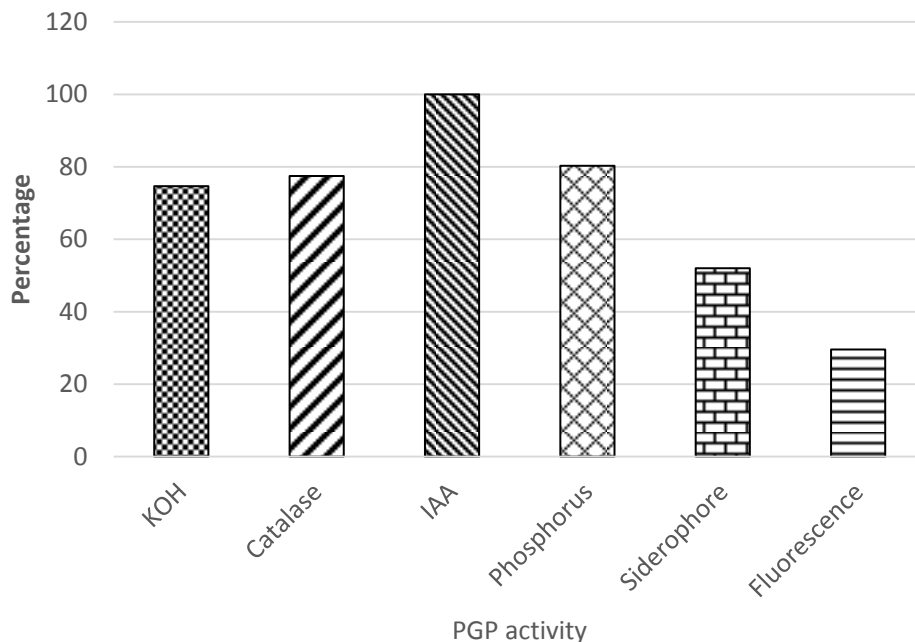
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production and secretion of substances of interest for plant development (Ahmad et al., 2008; Ndiaye et al., 2020; Pereira et al., 2020; Gouda et al., 2018). The results of this study show a large and diverse microbial community in the soil of the investigated site. Observation of bacterial colonies isolated from the soil reveals different types of colonies, some with similar characteristics and others with relatively variable ones. Thus, Gouda et al. (2018) reported a diversity of actions of rhizobacteria in the growth and development of plants in relation to their biodiversity in agroecosystems.

In the present study, a total of 71 isolates were selected on the basis of their phenotypic criteria. The results indicated that positive catalase activity was detected in 77.46% of the strains when the results of Bouras (2018) showed catalase activity of all isolated strains. This diversity of PGPRs could play a positive role in the growth and nutrient use efficiency of plants depending on their potential (Kumari et al., 2018b; Pereira et al., 2020) particularly in drought stress condition as Sahelian zones (Ahluwalia et al., 2021). Moreover, our study show that Gram-negative strains were more representative in the Sahelian soil (74.65%). This is correlated with the results of Kaioua and Grairi (2015), who reported most abundant Gram-negative

bacteria in the rhizosphere. These authors asserted also that the fluorescent bacteria belonged to *Pseudomonas* group. However, in contrast to these results, Kim et al. (2011) showed a more representativeness of Gram-positive strains in plant rhizosphere.

All isolated strains showed a positive reaction for auxin (IAA) production. In accordance to the founding of Kumari et al. (2018a), variable amounts of auxin were produced by the strains. For the amount of hormone production, our results corroborated those of Adouane et al. (2018) who obtained strains that produced auxin at various rates. However, Barazani and Friedman (1999) reported that bacteria secreting IAA level greater than 13.5 µg/mL are considered to be PGPR. Thus, it appears clearly that quantity of hormone production depends on the type of strain, and is not the same for all isolate indicating interest to screen isolates. Furthermore, the present study showed a large number of strains that were able to solubilize inorganic P at different degrees. These results are in accordance with those of Sharma and Roy (2005) who showed that bacteria solubilize P at different levels of efficiency depending on the type of isolate. Also, several works reported that plant growth is increased by siderophore-producing rhizobacteria through mineral nutrient acquisition and hindering growth of some



**Figure 5.** Relative importance of plant growth activity of isolated rhizobacterial strains.  
Source: Author

pathogenic microorganisms (Schalk et al., 2011; Amar et al., 2013). Our results showed on one hand, an important proportion of Gram-negative bacteria (53/71) and on the other hand 47.89% that have the capacity to chelate Fe in the CAS medium and considered as siderophores synthesizing rhizobacteria. Among these siderophore-producing strains 85.29% were Gram-negative. According to many authors, Gram-negative and -positive bacteria presented common genes involving in siderophore transport (Clarke et al., 2000; Schalk et al., 2011). Previous studies reported that fluorescent siderophore like pyoverdine is synthesized and secreted in plant environment and is generally produced by Gram-negative rhizobacteria classified in the genus *Pseudomonas* (Clarke et al., 2000; Das et al., 2020). In addition, our study showed a few presences of Gram-positive isolates that produced siderophores including some isolates able to fluoresce.

Due to the complexity of the phenomenon, the understanding of the mechanism of plant growth promotion by rhizobacteria is still under study. However, it is clear that effectiveness of PGPR as biofertilizer is no longer in doubt (Vessey, 2003; Canbolat et al., 2006). This justifies the expansion of its use throughout the world for the promotion of agro-ecology and organic agriculture (Amar et al., 2013; Das et al., 2020). In order to preserve the environment and to prevent the misuse of chemical fertilizers, a selection of strains with the best PGP traits is made for the production of effective biofertilizers. In particular, few investigations have been operated in PGPR population of the Senegalese Sahelian

semi- arid soils. One of the previous researches was done in the Northern agro-ecological zone of the Senegal River Valley (Ndiaye et al., 2020). According to the present study, the analyses of the importance of *in vitro* PGP effects of isolates revealed the existence of bacteria able to promote plant growth in soils of this zone (Figure 5).

Globally, among the 71 isolates, 77.46% have the capacity to detoxify the plant cells by destroying the excess hydrogen peroxide ( $H_2O_2$ ) produced and thereby prevent environmental stress (Martins and English, 2014; Bouras, 2018). All strains isolated are ability to synthesize phytohormones as IAA that play a major role in the regulation of plant growth and development as previously reported by several authors (Siddhi et al., 2014; Chitraselvi et al., 2015). At the same time, 80.28% of the strains can make P, an essential nutrient for plant growth, bioavailable in plant rhizosphere. Furthermore, an important proportion of isolates (52%) that produce siderophores can make the plant environment conducive to good development by inhibiting the growth of phytopathogens like fungi (de-Bashan, 2012). In addition, the 29.57% of fluorescent strains that synthesize pyoverdines are potential candidates for biocontrol of plant diseases and therefore plant bioprotectors. Synergic interactions of the PGP activities of rhizobacteria constitute a capital contribution on plant production. Therefore, a screening based on PCA of PGP activities and strains can be a useful tool for the selection of indigenous strains in order to propose a highly efficient biofertilizer for semi-arid Sahelian zone.

## Conclusion

This study aimed to detect good candidate to formulate biofertilizers for cowpea, groundnut and pennisetum to increase fodder plant production. Therefore 71 rhizobacteria strains are isolated from soils of the semi-arid area of Senegal. On the basis of phenotypic and plant growth-promoting traits, all isolates are characterized. Thereby, siderophore production and phosphate solubilization are not observed in all strains. However, they all have produced IAA even though the levels are relatively low. Some strains have shown both interesting IAA and siderophores production capacities. Nevertheless, analysis of correlations between the different PGP proprieties and strains hallow the selection of the best candidates to produce biofertilizer for improving crop growth.

## CONFLICT OF INTERESTS

The authors declare no conflict of interests.

## ACKNOWLEDGMENTS:

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