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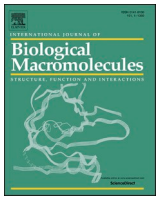

Controlled release of trifluralin herbicide using luminescent *Vibrio*-derived polyhydroxyalkanoate (PHA) microcapsules

Arefe N. Ahmadi [a], Ali Ganjeali [a], Mohammad Hassan Rashed Mohassel [b], Mansour Mashreghi [a,c,d,*]

[a] Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran
[b] Department of Agrotechnology, College of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran
[c] Industrial Biotechnology Research Group, Institute of biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran
[d] Nano Research Center, Ferdowsi University of Mashhad, Mashhad, Iran

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ABSTRACT

The controlled release of herbicides using new and safe materials can mitigate environmental pollution. Polyhydroxyalkanoate (PHA) is a type of biopolymer that can be produced by various bacteria. It has properties that make it suitable for encapsulation and controlled release applications. A luminescent bacterium, *Vibrio* sp. VLC strain was used as the PHA producer in this study. Initially, the polymer was synthesized by the bacterium following optimization of the culture medium, resulting in an approximate yield of 25 %. Subsequently, the produced polymer was analyzed using TEM, FTIR, and H-NMR techniques. Microcapsules were produced using the emulsion method. FE-SEM imaging revealed spherical microcapsules with an average diameter of 0.5–2 μm. The herbicide loading content and encapsulation efficiency were determined to be 16.64 % and 66.56 %, respectively. The herbicidal effect of the microcapsules containing trifluralin was investigated using *Amaranthus retroflexus* and *Setaria viridis* plants, demonstrating a significant reduction in various parameters after application. Furthermore, the impact of encapsulated herbicide on soil microbial population was assessed, revealing a less negative effect compared to its free form. These findings suggest that the PHA from a luminescent vibrio holds promise as an eco-friendly, biodegradable, nontoxic material for the controlled release of herbicides.

1. Introduction

In agriculture, herbicides are commonly used for weed control to eliminate the competition with crops, thereby increasing crop yields. Currently, herbicides account for about 60 % of the total consumption of agrochemicals, making most large-scale agricultural products heavily dependent on them [1,2]. Herbicides are classified into several categories based on their functions in the target plant as they can target various plant systems. Inhibition of each of these systems disrupts plant growth and leads to its destruction [3,4]. Over the years, instances of herbicide resistance have been reported. This resistance often arises from mutations in the herbicide's target sites [5,6]. Therefore, sometimes herbicides are used much more than the required dose to destroy the target plant, leading to various problems, such as environmental pollution which is a potential health risk to different kinds of living organisms [7–10].

However, herbicides are one of the most effective and important tools in the management of weeds in agriculture, and therefore appropriate solutions must be applied to use these materials suitably with reduced risk [11]. Among the solutions studied is controlled release technology (CR). One of the important applications of controlled release systems is drug delivery in the medical field [12]. In agriculture, this technology offers various advantages other than gradual herbicide release such as increasing the duration of the herbicide effect compared to the same volume used in conventional methods, reducing the negative effect of herbicide on other plants, preventing evaporation or leakage to the lower layers of the soil, protecting the herbicide against decomposition factors like heat and light. This technology allows for the use of agrochemicals in reduced amounts without compromising their properties. This means reducing costs in addition to reducing some negative effects such as the leakage of chemicals into the surrounding environment [13–16].

So far, various materials have been investigated for the controlled release of herbicides using encapsulation method including materials

* Corresponding author at: Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran.
E-mail address: mashrghi@um.ac.ir (M. Mashreghi).

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such as starch [17], alginate/cellulose [18], chitosan [19], polylactic acid [20], polycaprolactone [21], chitosan/pectin/tripolyphosphate [22], polyhydroxyalkanoate and silica [23]. Polyhydroxyalkanoates (PHAs) have received a lot of attention for controlled release systems. In recent years, due to the advantageous properties of PHAs such as biodegradability, biocompatibility, high temperature stability, elasticity and resistance to UV, they have been widely studied and used in various fields, including agriculture, medicine, and the manufacturing of different materials. These materials also can be used for bioplastics production, tissue engineering and drug delivery systems [24,25]. This family of polymers is polyester of hydroxyalkanoates, which are produced by many microorganisms as a carbon and energy source in the form of intracellular granules [26–31]. Different bacteria exhibit varying yields of PHA production, with some, such as *Cupriavidus necator*, demonstrating particularly high levels of PHA synthesis [32–36]. The PHA polymer have been produced using several species of *Vibrio* such as *Vibrio azureus* [37], *Vibrio harveyi* [38], *Vibrio proteolyticus* [39] and *Vibrio alginolyticus* [40]. Marin bacteria such as *Vibrio* are mostly halophilic and highly tolerant to salt and for this reason, the probability of medium contamination is low for these bacteria. This can be important in large scale PHA production as it can be more cost effective to lower the contamination [40–42]. Biodegradability of PHAs in soil can vary based on soil properties such as temperature, moisture content, and microbial composition, as well as the characteristics of the polymer itself. Some studies indicate that PHA-based materials similar to those used in this study can degrade up to 100 % in soil within approximately a hundred days. This demonstrates that PHA will largely be removed from the environment following its intended application, which, in this case, is the gradual release of herbicide in a stable manner [43].

Trifluralin is in the category of dinitroaniline herbicides. It is a pre-emergence selective herbicide suitable for controlling wheatgrass and broadleaf grasses and is commonly used in various crops fields like sunflowers and fruit trees or non-agricultural plants like ornamental grasses. Because of its physiochemical properties like high volatility and persistence in environment, trifluralin can easily leach into water and cause contamination in the environment [44–46]. Research on PHA derived from *Vibrio* species primarily focuses on optimizing production processes to maximize yield. In this study, after optimizing PHA production using *Vibrio* sp. VLC, we aimed to explore the application of the produced PHA in agriculture. Our goal was to determine whether the polymer derived from this marine bacterium can be utilized similarly to PHA extracted from more widely recognized producers, such as *Cupriavidus necator*. It is noted that PHA from marine bacteria may exhibit different properties compared to those produced by other bacterial sources [47–52]. After developing microcapsules using PHA polymer to encapsulate trifluralin herbicide, the characteristics of these microcapsules as well as their effects on model plants and soil bacteria were investigated.

2. Materials and methods

2.1. Bacterial strain and chemical agent

Vibrio sp. VLC was obtained from a shrimp breeding pond located in southeast Iran (Goater Island) during previous studies. Upon sequencing the 16S rRNA gene, it exhibited a 100 % match to *Vibrio harveyi* [53]. Trifluralin herbicide (48 % purity) was obtained from Ariashimi company (Iran).

2.2. Polyhydroxyalkanoate production

Bacteria were cultured in two steps. First, a modified nutrient broth culture medium was used to prepare the inoculum by cultivating *Vibrio* sp. VLC. Sea salt (2 g/L) and glycerol (0.2 mL/L) were added to the nutrient broth culture medium. Then, the medium was inoculated with bacteria and incubated in a shaker incubator at 28 °C at 150 rpm (Vision Scientific VS-37S series, South Korea). After 24 h, 1 % of this medium was used for inoculation in the polymer production culture medium.

In second step, a modified nutrient broth medium was used to produce polyhydroxyalkanoate (PHA). To optimize the cultivation conditions for maximum polymer production, a Taguchi experimental design method using Minitab 21 software was applied [54]. The bacteria were cultivated in nutrient broth supplemented with glycerol and sea salt under various conditions (different glycerol and sea salt percentages and incubation time) and then incubated in a shaker incubator at 28 °C at 150 rpm (Table 1).

2.3. Polyhydroxyalkanoate extraction

Polymer extraction was performed using the method of Yang et al. [55]. First, 0.5 g of lyophilized bacteria were treated with 5 % sodium dodecyl sulfate (SDS) solution in a water bath at 60 °C for 3 h. The resulting solution was centrifuged (10,000 rpm for 10 min) and precipitates (polymer) were rinsed with distilled water several times. The extracted polymer was then freeze-dried for 24 h (Christ, Germany).

2.4. Polyhydroxyalkanoate characterization

The extracted polymer was mixed with potassium bromide (KBr) and subjected to Fourier Transform Infrared Spectrophotometer (Thermo-Nicolet AVATAR 370 FTIR, USA) with a spectrum recorded between 4000 and 400 cm^{-1}. For Proton Nuclear Magnetic Resonance Spectroscopy (H-NMR), 10 mg of dried polymer was dissolved in chloroform and analyzed using Bruker Avance III spectrometer operated at 300 MHz. The morphology of bacteria and the polymer granules were observed using a transmission electron microscope (TEM) (ZEISS LEO 912 AB, Germany). For TEM imaging, a bacterial culture was utilized with 1 % glycerol, 2 % salt, and an incubation time of 48 h, as this combination was shown to yield the highest amount of PHA. The control sample was prepared under identical conditions, with the exception of glycerol.

2.5. Preparation of microcapsules

Emulsion method was used to prepare microcapsules [56]. In this method, the aqueous phase was prepared by dissolving polyvinyl alcohol (PVA) in water at a 1 % ratio. The oil phase contained a 2 % (*w/v*) PHA solution and Trifluralin (purity of 48 %) in chloroform. Microcapsules were loaded with 25 % (w/v) of herbicide and the volume ratio of aqueous phase to oil phase was 5 to 1. The oil phase was added dropwise into the aqueous phase and mixed at 800 rpm using a magnetic stirrer. After 24 h, the microcapsules were centrifuged (10,000 rpm for 10 min), rinsed with deionized water and freeze-dried. The microcapsules were observed using an optical microscope (Olympus-Japan) and a field emission scanning electron microscope (LMU TESCAN BRNO-Mira3) to examine their morphology. For determining particle size, trifluralin microcapsules were analyzed using Dynamic Light Scattering (DLS) technique (Horiba-SZ100 – Japan). The analysis was conducted using an aqueous solution of microcapsules at a scattering angle of 90° and a temperature of 25 °C.

2.6. Loading content and encapsulation efficiency

Microcapsules (25 mg) were dispersed in 20 mL of methanol and treated by ultrasonic vibration for 30 min. After centrifugation (10,000 rpm for 3 min), the supernatant was collected and the concentration of herbicide was measured using a spectrophotometer (Nanodrop Array Spectrophotometer - Nano Ar 2015) at the wavelength of 220 nm. The loading content and encapsulation efficiency were calculated using Eq. 1 and 2 *[57]*.

Table 1
levels and factors designed according to Taguchi method (exp: experiment number, G: Glycerol (*v*/v%), S: Sea Salt (*W*/*v*%), t: Incubation time (hour).

exp.	G	S	t	exp.	G	S	t	exp.	G	S	t
1	1	1.5	24	4	1.5	1.5	24	7	2	1.5	24
2	1	2	48	5	1.5	2	48	8	2	2	48
3	1	2.5	72	6	1.5	2.5	72	9	2	2.5	72

$$LC = \frac{Wt}{Wmc} \times 100 \quad (1)$$

$$EE = \frac{Wt}{IWt} \times 100 \quad (2)$$

In these equations, LC and EE represent the herbicide loading content and encapsulation efficiency, respectively. Also, Wt, Wmc and IWt indicate the weight of the herbicide in the microcapsule, the weight of the microcapsule and the initial weight of the herbicide.

2.7. Herbicide release rate from microcapsules

Microcapsules (50 mg) were dissolved in 10 mL of water and treated by an ultrasonic device. The solution was transferred into a dialysis bag and immersed in 50 mL of 10 % methanol aqueous solution (release medium). It was placed in a shaker incubator and homogenized the release medium at 100 rpm. At time intervals of 6, 12, 24, 48, 96 and 144 h, 2 mL of the medium was removed and replaced with an equal amount of a 10 % methanol solution to maintain a constant volume of the release medium. The concentration of herbicide in the samples was measured by a spectrophotometer at the wavelength of 220 nm. After obtaining the concentration of the herbicide at certain times, the release rate of the herbicide was calculated.

2.8. Herbicidal activity of microcapsules

Herbicidal activity of trifluralin loaded microcapsules was studied using *Amaranthus retroflexus* (broad-leaf plant) and *Setaria viridis* (narrow-leaf plant) as model plants. These plants have a rapid growth rate and grow in various agricultural fields. The herbicidal activity of encapsulated herbicide was compared to its free form against model plants. Microcapsules containing herbicide (shown as MCT) and empty microcapsules (shown as MC) were added to the soil surface at the same time as the seeds were planted. For positive control (shown as C+) soil was treated with commercial formulation of trifluralin. Pots with no herbicide were used as the negative control (shown as C-). In all experiments, 15 seeds of the model weeds were planted and the pots were placed in a greenhouse maintained at room temperature.

To evaluate the efficiency of microcapsules, the fresh weight and height of the aerial parts of the plants were measured after 20 days. Also, the number of plants in each treatment were used to calculate the germination percentage (GP) and germination rate index (GRI). (Eqs. 3 and 4 presented in Table 2 with explanations).

2.9. Effect of microcapsules on soil bacteria

Soil bacterial population was examined using the colony forming unit method (CFU) to determine the effect of each treatment on them. For this purpose, soil samples were collected at regular time intervals of 5 days. After that, each sample was diluted and cultivated in nutrient agar plates and then incubated at 37 °C. Bacterial colonies in each plate were counted after 48 h.

Table 2
Equations used for calculating GP (germination percentage) and GRI (germination rate index) for the model plants.

Eq. number	Eq.	Eq. components
3	$GP = \frac{N_g}{N_t} \times 100$	N_g: number of germinated seeds N_t: Total seeds
4	$GRI = \frac{G_1}{1} + \frac{G_2}{2} + \ldots + \frac{G_i}{i}$	G_i: germination in each day i: day after cultivation

3. Results and discussions

3.1. PHA production and extraction

The optimization of bacterial culture medium conditions was achieved by varying three factors: the percentage of glycerol, salt concentration, and incubation time. This optimization process involved conducting nine experiments designed using the Taguchi method within Minitab 21 software. Subsequently, bacterial polymer extraction was carried out for each experiment. The amount of bacterial biomass and extracted polymer were then measured for each experiment, and the resulting data was plotted accordingly (Fig. 1A).

The results showed that the highest biomass yields were observed in experiments 2 (1.449 g/L) and 9 (1.405 g/L), while the highest amount of polymer was obtained in experiments 2 (24.72 %) and 7 (18.275 %). These findings suggest that while biomass levels can affect the amount of polymer production (as polymers are synthesized within bacterial cells), various other variables can also influence polymer production. For instance, in experiment 9, despite high biomass levels, factors like salt percentage, glycerol concentration, and incubation duration examined in this study may have led to a reduction in polymer synthesis. The Duncan method was employed to compare mean values in all the experiments, revealing no significant differences in biomass quantities. However, in case of polymer production, experiments 2 and 7 exhibited significant distinctions from each other and from the remaining experiments.

Utilizing Taguchi analysis, the optimal quantities of glycerol percentage, salt concentration and incubation time for biomass production were determined to be 1 %, 2.5 % and 48 h respectively. For polymer production, these optimal quantities were 1 %, 2 % and 48 h respectively (Fig. 1B and C). These results revealed that maximizing biomass is not necessarily followed by maximized polymer production. In this study, the 2.5 % salt concentration, which favored biomass production, did not yield the same results for polymer production. This suggests that factors like glycerol percentage and incubation time may exert a more pronounced influence on polymer production than biomass quantity. Furthermore, the analysis indicates that among the variables tested, incubation time had the most significant impact on biomass production, while glycerol and salt percentages had little effects. In contrast, all three factors including glycerol percentage, salt percentage, and incubation time, significantly influenced polymer yield. Notably, the impact of salt concentration was more pronounced compared to the other factors. The interactions between all three factors showed that the most significant interaction affecting biomass production occurs between the quantities of glycerol and salt (Fig. 1D and E). In case of polymer production, a notable level of interaction is observed among all factors, suggesting their collective influence on polymer yield [58,59]. In summary, the optimal conditions for biomass and PHA production differ, with incubation time being the most critical factor for biomass and salt concentration having the greatest impact on polymer yield. The interactions between the factors also vary, with glycerol and salt concentrations significantly influencing biomass production, while all three factors collectively impact PHA yield [60].

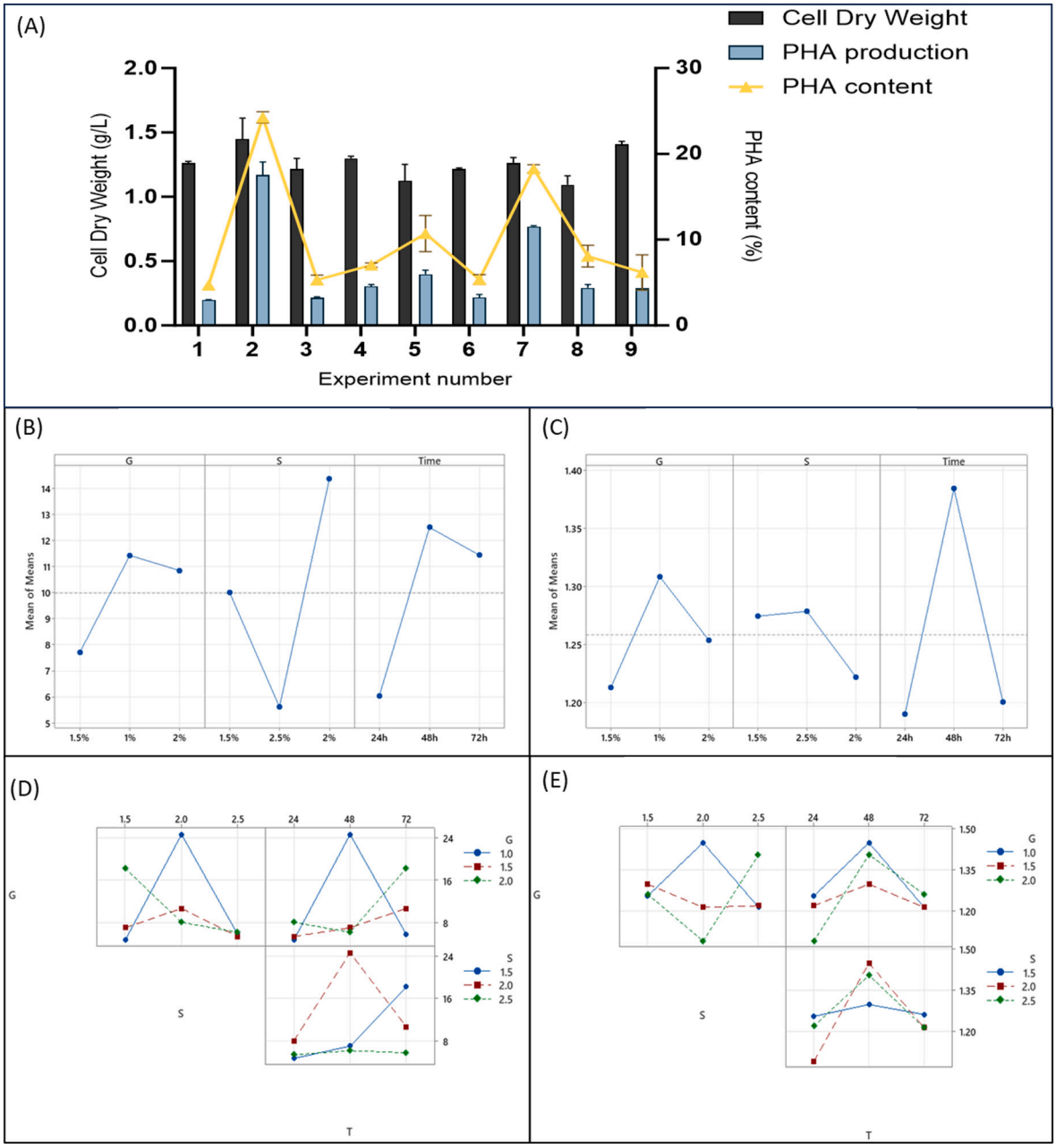

Fig. 1. A. Biomass and PHA production in *Vibrio* sp. VLC in different experiments designed by Taguchi method. B. The impact of glycerol percentage (G), salt concentration (S) and incubation time (T) on polymer production and C. biomass production. Interaction plot between the three parameters in D. polymer production and E. biomass production based on Taguchi analysis.

The results highlight the complex relationship between biomass production and PHA synthesis in bacterial cultures. While biomass is a prerequisite for polymer production, as it provides the cellular machinery and resources for polymer synthesis, other factors such as nutrient composition, environmental conditions, and incubation parameters can also significantly influence the efficiency of PHA production. [61,62]. The findings suggest that optimizing both biomass production and polymer synthesis is crucial for enhancing the overall yield and productivity of bacterial polymer production systems [63].

3.2. TEM imaging

The produced PHA polymer appeared as distinct bright spots within the bacterial cell (Fig. 2A and B). These bright spots were also visible in the control sample, although in fewer abundance compared to the sample cultured in a glycerol-containing medium. These results indicate that while the PHA polymer was synthesized even in the absence of glycerol, its production yield was notably lower compared to the sample grown in a carbon-rich medium. Several studies have reported the abundance of bright spots correlates with the PHA production yield in which more spots indicate higher production [64,65]. In addition, the higher abundance of white spots in the glycerol-containing sample indicates that glycerol enhanced the PHA production yield compared to the control sample without glycerol [66].

3.3. Polymer characterization

FTIR analysis of the extracted polymer revealed peaks in 1638.23 cm-1, 1112.3 cm-1, 1323.89 cm-1 and 3444.24 cm-1, corresponding to the C═O, C-O-C, CH3 and OH bonds, respectively (Fig. 2C). These peaks

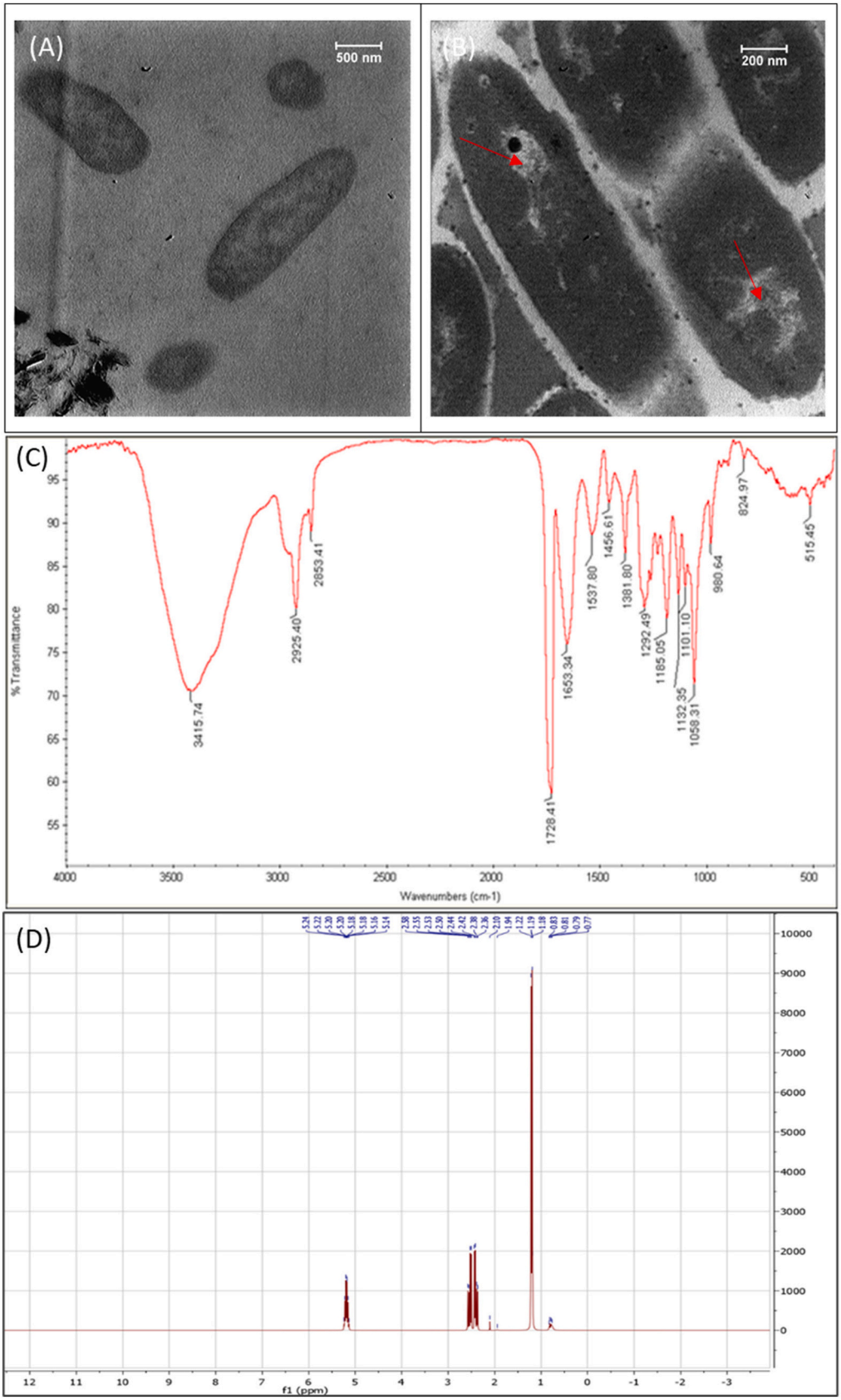

Fig. 2. A. TEM imaging of control sample of bacteria and B. bacteria cultured in 1 % glycerol, arrows indicate the site in which PHA produced. C. FTIR spectrum and D. H-NMR analysis of the extracted polymer from *Vibrio* sp. VLC.

in the polymer's spectrum confirm its identity as a PHA. Additionally, the presence of an ester carbonyl group at 1728 cm-1 indicates that the polymer is a short-chain PHA. The H-NMR analysis of the extracted polymer displayed peaks at 1.22 ppm, 2.5 ppm, and 5.22 ppm, corresponding to methyl, methylene, and methane groups, respectively (Fig. 2D). Comparison results with previous studies by various authors supports the identification of the produced polymer as polyhydroxybutyrate (PHB), a type of short-chain PHA.

The C═O and C-O-C peaks in the FTIR spectrum are characteristic of the ester bonds in the PHA backbone, while the CH_3 and OH groups are associated with the side chains and terminal hydroxyl groups, respectively. The ester carbonyl peak at 1728 cm^{-1} specifically indicates a short-chain PHA structure. The H-NMR peaks at 1.22 ppm (methyl), 2.5 ppm (methylene), and 5.22 ppm (methane) are consistent with the reported values for PHB. The FTIR and H-NMR analyses, in conjunction with comparisons to previous studies [37,38,67,68], confirm that the extracted polymer is PHB, produced by the bacterial cells under the given growth conditions.

3.4. Microcapsule preparation

Trifluralin herbicide was successfully encapsulated in microcapsules using the emulsion method. The resulting microcapsules were obtained as a yellow powder after solvent removal and subsequent drying in a freeze-dryer. The morphology of the microcapsules was examined using both an optical microscope and a field emission scanning electron microscope (FE-SEM) to determine their structure (Fig. 3).

The resulting microcapsules were found to be spherical with a relatively smooth surface and a diameter of approximately 0.5 to 2 μm. The nearly uniform-sized spherical microcapsules produced by the dropwise mixing of the two phases in the preparation process demonstrate that the emulsion method can effectively be used to create spherical trifluralin microcapsules.

The particle size determined by DLS analysis was found to range from 500 nm to 1 μm, which is relatively consistent with the FE-SEM images. Prudnikova et al. showed that PHA can be used effectively to construct environmentally friendly sustained-release PHA-herbicide biodegradable microgranules that can be placed into the soil together with seeds [69]. They indicated that the material used to prepare carriers for herbicide must have such properties as bio-degradability, environmental safety, long-term retention in nature and controlled degradation to nontoxic products.

3.5. Loading content and encapsulation efficiency

The herbicide loading content and encapsulation efficiency were assessed by dissolving a specific quantity of PHA microcapsules in methanol. Subsequently, the solution was centrifuged, and the absorbance of the supernatant was measured at 220 nm using a spectrophotometer. The loading content was determined to be 16.64 %, the encapsulation efficiency was found to be 66.56 %. Mohammed et al. used pollen-derived microcapsules for the encapsulation of aspirin and only achieved a loading capacity of 26.7 % and an encapsulation efficiency of 53.4 % [70]. In a similar study on the encapsulation of the herbicide, the loading content under optimized conditions was determined to be 16.50 %, which is very close to the results we obtained [57]. Therefore, the choice of materials, such as biodegradable natural biopolymers, can significantly influence the loading content and the encapsulation performance of herbicide-loaded microcapsules, improving herbicide efficacy and reducing the potential hazardous environmental impact.

3.6. Herbicide release rate

The trifluralin release rate was determined by measuring the herbicide amount in the release medium at various time intervals (Fig. 4).

Initially, within the first two days, the herbicide exhibited a rapid release from the microcapsule, likely due to its faster diffusion from the surface or near-surface regions of the microcapsule. This initial burst release is a common phenomenon in controlled release systems, where the herbicide molecules closest to the surface of the microcapsule [71]. Following this initial period, after 48 h, a noticeable decrease in the release rate was observed as indicated by a reduction in the slope of the curve. This decrease in release rate suggests that the initial rapid release phase has ended, and the herbicide is now being released at a slower,

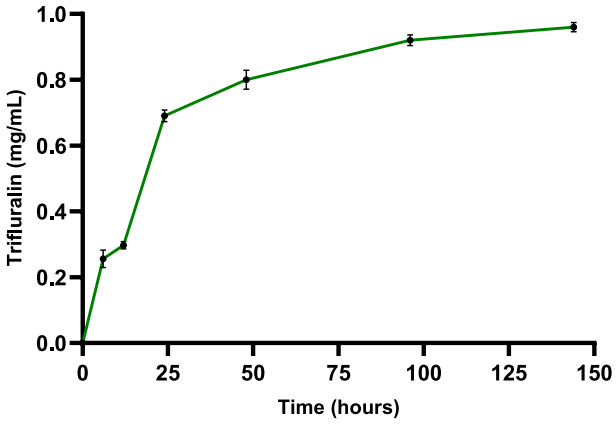

Fig. 4. Release rate of herbicide from PHB microcapsules after 6, 12, 24, 48, 96 and 144 h.

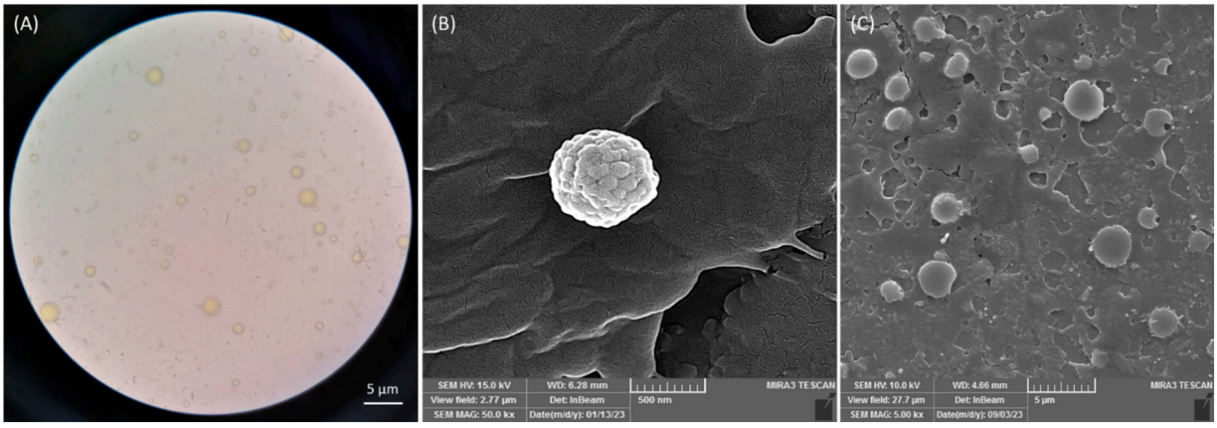

Fig. 3. A. Light microscopic image of microcapsules (x100). B. FE-SEM image of a single microcapsule and C. FE-SEM image of microcapsules.

more controlled rate. Subsequently, a gradual and relatively constant release rate of the herbicide was observed. This type of gradual herbicide release has also been observed in other studies that utilized PHA as a herbicide carrier [57,72]. These findings validate the controlled and gradual release of trifluralin herbicide from the microcapsules developed in this study, demonstrating their potential for use in agricultural applications where sustained herbicide release is beneficial [73].

3.7. Herbicidal effect of microcapsules

The impact of microcapsules containing the herbicide trifluralin on *Amaranthus retroflexus* and *Setaria viridis* plants was studied following a 20-day incubation period. Plant height and fresh weight were measured, while germination percentage (GP) and germination rate index (GRI) were determined based on the germinated plant count in each pot (Fig. 5). For *Amaranthus retroflexus*, among the 15 planted seeds in each pot, the highest number of germinated plants was observed in the control pots without herbicide (C-) and the pots containing empty microcapsules (MC), with an average of 13 to 14 germinated plants (Fig. 5A). This number decreased to approximately 5 germinated plants in the positive control pots containing the herbicide without microcapsules (C+), while the lowest count was found in the pots containing microcapsules loaded with trifluralin (MCT), with an average close to 1 germinated plant. The results show that the microcapsules effectively delivered the herbicide trifluralin, leading to a significant reduction in the germination of *Amaranthus retroflexus* seeds compared to the positive control. The empty microcapsules did not show any adverse effects on seed germination, indicating the safety of the microcapsule material

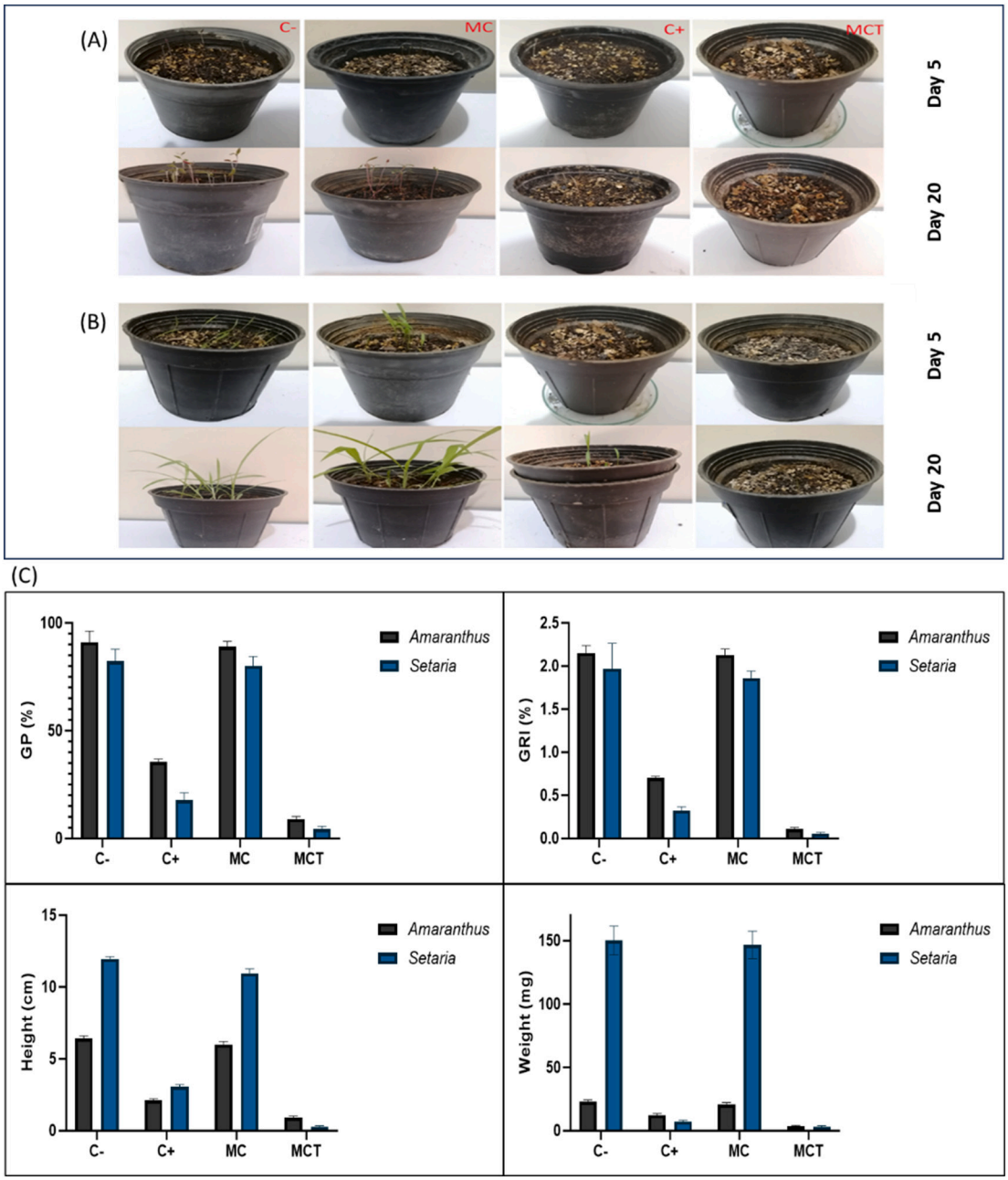

Fig. 5. The effect of different treatments on the growth of target plants after 20 days in A. *Amaranthus retroflexus* and B. *Setaria viridis*. C. The quantified effect of each treatment on different parameters in the model plants. The treatments included negative control (C-), positive control (C+), microcapsule without herbicide (MC), and herbicide-containing microcapsule (MCT). GP and GRI indicate germination percentage and germination rate index, respectively.

itself [74].

The variables associated with seed germination, including germination percentage (GP) and germination rate index (GRI), exhibited a consistent trend across different treatment groups (Fig. 5C). The GP for the control group without herbicide (C-) was approximately 91 %, while for the group with empty microcapsules (MC), it was around 89 %. In contrast, the germination percentage decreased to 35 % in the positive control group with the herbicide trifluralin (C+) and further reduced to 9 % in the group treated with trifluralin-loaded microcapsules (MCT). Similarly, the germination rate index (GRI) showed a comparable trend. The GRI for the control group (C-) and the group with empty microcapsules (MC) was around 2 % per day. However, the GRI decreased to 0.7 % per day in the positive control group (C+) and further declined to 0.1 % per day in the group treated with trifluralin-loaded microcapsules (MCT). The consistent reduction in both germination percentage and germination rate index from the control groups to the trifluralin-treated groups, particularly in the microcapsule-treated group (MCT), proposes that the trifluralin-loaded microcapsules effectively inhibited seed germination. Therefore, the development of new herbicide formulations using microcapsules can be an effective approach for weed control [75].

Plant height and weight measurements revealed that the control (C-) and empty microcapsule (MC) samples exhibited the highest values, while the positive control (C+) and trifluralin-loaded microcapsule (MCT) samples showed lower measurements (Fig. 5C). The heights of the C-, MC, C+, and MCT samples were approximately 6.5, 6, 2, and 1 cm, respectively. Regarding weight, the C-, MC, C+, and MCT samples weighed around 22.5, 20, 12, and 3.5 mg per pot, respectively.

Statistical analysis using Duncan's test revealed that microcapsules without herbicide (MC) and the negative control (C-) exhibited similar results in terms of plant growth and seed germination. However, microcapsules containing herbicide (MCT) demonstrated a greater inhibitory effect on plant growth and seed germination compared to the positive control (C+) containing the free herbicide. The only exception was plant weight, where MC and C+ samples showed statistically similar results. Therefore, it was determined that microcapsules containing herbicides were more effective than free herbicides. On the other hand, microcapsules without herbicides showed no impact on plant growth, indicating that the sole PHA polymer does not have any specific adverse effects on plants [76,77].

The results for *Setaria viridis* showed a significant difference in plant growth and germination variables among the treatment groups (Fig. 5B and C). The control (C-) and microcapsules without herbicide (MC) samples exhibited the highest number of plants, plant height, and plant weight. Specifically, out of 15 planted seeds, 12 germinated in both C- and MC pots, while only 3 and 1 germinated in the positive control (C+) and microcapsules with herbicide (MCT) pots, respectively. In terms of plant height, the C- and MC samples reached an average height of 12 cm and 11 cm, respectively, while the C+ and MCT samples only reached 3 cm and 0.2 cm, respectively. Similarly, the plant weights after the test period were significantly higher for the C- and MC samples, with average weights of approximately 150 mg and 146 mg, respectively, compared to 7 mg and 3 mg for the C+ and MCT samples, respectively. Therefore, the presence of herbicide in the microcapsules (MCT) had a significant inhibitory effect on *Setaria* growth and germination, while the microcapsules without herbicide (MC) did not affect plant growth compared to the control (C-).

Duncan's test analysis revealed slight variations in the results obtained for the *Setaria* plant compared to *Amaranthus*. In the MCT sample of *Setaria*, there were no significant differences observed in the number of plants, plant weight, GP, or GRI rates when compared to the C+ sample. However, a notable variance was found in plant height between the MCT and C+ samples. Similar to *Amaranthus*, there were no significant disparities in the performance of MC and C- treatments. These findings suggest that, for the most part, there is no significant difference between microcapsules containing herbicides and free herbicides, except for plant height, where MCT exhibited superior performance over C+ sample.

The impact of microcapsules containing herbicide (MCT) on each plant was assessed by quantifying the decrease in various parameters. The findings revealed that in *Amaranthus* pots, the height, fresh weight, germination percentage, and germination rate index decreased by approximately 85.5 %, 83.5 %, 90 %, and 95 %, respectively, compared to the herbicide-free sample (C-). In *Setaria*, the reductions were slightly more pronounced, with approximately 98 %, 98 %, 95 %, and 97.5 % decreases observed in height, weight, germination percentage, and germination rate index, respectively. Comparison of these results to C+ samples showed that encapsulated herbicide had a better effect on both plants (Fig. 6).

After analyzing the results, it was observed that in all cases, the decrease in the measured factors in the *Setaria* plant exceeded that of the *Amaranthus* plant. Utilizing an independent *t*-test, this difference was found to be statistically significant (P value <0.05) specifically in relation to plant height and weight. In other words, the herbicide-containing microcapsule demonstrated a similar impact on both plants across all parameters except for plant height and weight. Notably, with regards to these two factors, the treatment had a more pronounced effect on *Setaria* (narrow leaves) plants, showing a significant difference compared to *Amaranthus* (broad leaves). The results of this experiment were compared with similar studies that utilized PHA and other materials, such as lignin and chitosan, for the controlled release of various herbicides. These studies examined the herbicidal effects of microcapsules containing herbicides, which were found to be comparable to or more effective than free herbicides. Notably, some studies reported a reduction in weed growth of over 90 % following treatment with encapsulated herbicides which is in alignment with the findings in our research [23,57,69,78–83].

3.8. Effect of different treatments on soil microbial population

Numerous studies suggest that various agrochemicals, including herbicides, can negatively impact on the soil microbial populations due to their chemical composition, frequency of application and soil properties like pH and moisture levels. This disturbance disrupts the natural balance within these populations, potentially causing changes in the surrounding ecosystem. For example, studies suggest that the decline in plant life following herbicide application may be linked to the loss of beneficial symbiotic bacteria like rhizobia. Moreover, the continuous application of herbicides can intensify their impact on microbial communities over time. Therefore, management of using such herbicides is essential to mitigate these repercussions [84–87].

In this study, the impact of each treatment on the soil microbial population was examined through soil sampling conducted on days 5, 10, 15, and 20. The experiment aimed to investigate the efficacy of microcapsules in maintaining the balance of microbial populations compared to free herbicides. Following dilution of each sample in physiological saline solution, they were cultured on nutrient agar plates. The soil microbial population was quantified by counting the colony forming units. The findings revealed a reduction in soil microbial populations upon the introduction of herbicides, whether in the free form or as microcapsules. Furthermore, the graph illustrating the free herbicide displayed a lower level in contrast to the herbicide encapsulated within microcapsules (Fig. 7).

Duncan's test was employed to conduct a comprehensive comparison of the results. The data was separately analyzed on different days (Table 3). Initially, during the first day of experiment, the soil microbial populations in all samples displayed similar levels. However, in the following days, a significant decrease in the microbial population was noted in the C+ sample compared to the other samples. In relation to the herbicide-containing microcapsule, despite showing similar performance to C+ on two days of the experiment, the microbial population exhibited closer alignment with C- and MCT samples on other days. This indicates a less severe negative impact of encapsulated herbicide on soil

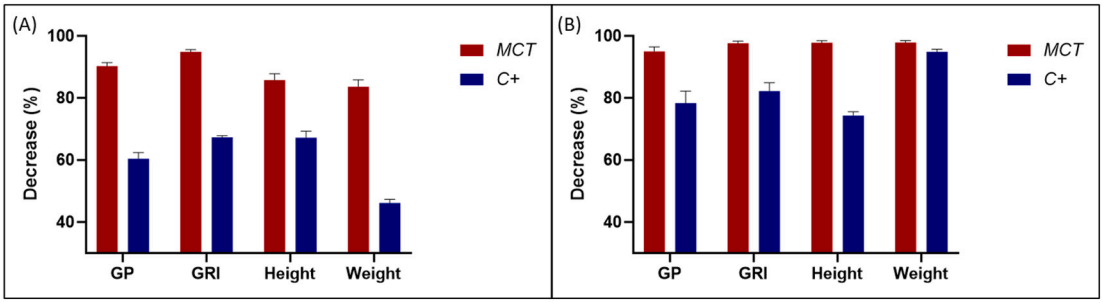

Fig. 6. The effect of microcapsules containing herbicide on the morphological characteristics and variables related to germination in A. *Amaranthus* and B. *Setaria* compared to positive control. GP and GRI indicate germination percentage and germination rate index, respectively. Decrease% was calculated after comparison with negative control.

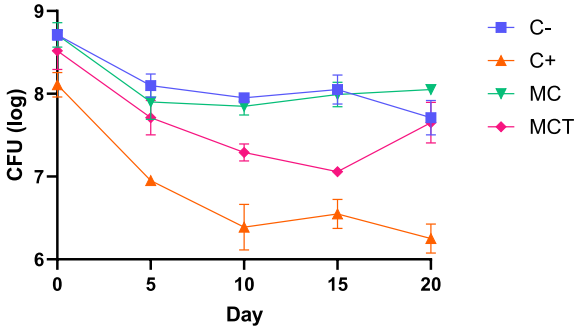

Fig. 7. The soil microbial population was analyzed during the experimental period (20 days) in different treatments: C-, C+, MC, and MCT, which represent negative control, positive control, microcapsules without herbicide and herbicide-containing microcapsule, respectively. The results were statistically analyzed using Duncan's test, and significant differences are shown in Table 3.

Table 3
Significance letters related to microbial population on various days according to Duncan's test. Samples sharing the same letter are not significantly different. C-, C+, MC, and MCT correspond to negative control, positive control, microcapsules without herbicide and herbicide-containing microcapsule, respectively.

	MCT	MC	C+	C-
Day 1	a	a	a	a
Day 5	ab	a	b	a
Day 10	a	a	b	a
Day 15	b	a	b	a
Day 20	a	a	b	a

microbial populations when compared to free herbicide.

The results were compared with prior studies which extensively investigated the influence of encapsulated herbicide and free herbicide on soil microbial communities. Their investigations indicated that initially, there was no noticeable difference in the impact of free herbicide compared to encapsulated herbicide on soil microbial populations. However, over time, the adverse effects of free herbicide intensified. Ultimately, encapsulated herbicide demonstrated superior performance in preserving the balance of soil microbial populations [69,88]. The findings of this study partially corroborated the aforementioned research. Moreover, as previously mentioned, the conventional application method of herbicide requires frequent reapplication in large quantities, whereas the encapsulated herbicide with controlled release properties can be applied less frequently in smaller doses. Consequently, it is plausible that over an extended period, the negative impact of the conventional herbicide on soil microorganisms may even surpass that of the encapsulated herbicide even compared to initial days of application.

4. Conclusions

The objective of this study was to achieve controlled release of trifluralin herbicide by encapsulating it within PHA polymer produced by *Vibrio* sp. VLC with a yield of 25 % of cell dry weight. Compared to free herbicide, encapsulated herbicide had a greater impact on *Setaria*, while showing a similar effect to free form on *Amaranthus* with about 90 % decrease in germination percentage in both plants compared to negative control. Furthermore, the encapsulated herbicide had a milder negative effect compared to free herbicide on soil microbial population. The study shows that PHA extracted from *Vibrio* sp. VLC is effective for herbicide encapsulation, comparable to PHA from other bacteria. Additionally, using *Vibrio* for PHA production reduces contamination risk due to the high salt concentration in the culture medium, making it suitable for large-scale production. Overall, the research findings highlighted the benefits of utilizing herbicide encapsulated in PHA biopolymer produced by bacteria over conventional method of free form of herbicide. Given that this study was conducted on a small scale in a greenhouse with controlled environmental conditions, further research is needed to assess the effects of trifluralin microcapsules at larger scales. The behavior of microcapsules in field conditions, where environmental parameters fluctuate, may differ from that observed in the greenhouse.

CRediT authorship contribution statement

Arefe N. Ahmadi: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Ali Ganjeali:** Writing – review & editing, Visualization, Validation, Project administration, Methodology. **Mohammad Hassan Rashed Mohassel:** Visualization, Validation, Project administration, Methodology. **Mansour Mashreghi:** Writing – review & editing, Validation, Supervision, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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