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Article

Effect of using sweet whey in irrigation of alfalfa and corn

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Abstract

The use of whey in agricultural applications is an option to reduce the environmental impact it causes when it is discharged without control. The purpose of this work was to measure the phytotoxicity of sweet whey that is generated during the production of fresh cheese in seeds and seedlings of alfalfa (*Medicago sativa* L.) and corn (*Zea mays* L.) as test-target organisms. Four individual experiments were carried out in the Laboratory of Microbial Molecular Ecology of the CICM-ICBUAP in the city of Puebla, Mexico, from June to December 2018 and January 2019, under controlled conditions, both in Petri dishes with culture medium and in seedling trays with substrate, in a completely randomized experimental design (CRED) with five treatments (4, 8, 12, 16 and 20% whey and a drinking water control). The variables evaluated were the lethal concentration (LC_{50}), by inhibition of germination in the two seeds, and the sublethal concentration (SLC), by decreased development of sprouts and seedlings. The results obtained indicate that sweet whey has an LC_{50}-5 of 53% and an SLC-10 of 12% in alfalfa seeds ($p< 0.05$) and an LC_{50}-60 of 20% in alfalfa seedlings and an LC_{50}-20 of 20% in corn seedlings. At 60 days, it was observed that, at concentrations between 4 and 6%, sweet whey has a growth-promoting effect on alfalfa seedlings ($p< 0.05$), concluding that it is possible to use diluted whey in sustainable agricultural practices safely.

Keywords:

germination, phytotoxicity, seedlings.

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Introduction

In the cheese industry, 100 L of processed milk yields between 85 and 90 L of whey with retention of up to 55% of milk nutrients (Utama *et al*., 2017; Montalvo-Salinas *et al*., 2018). Globally, between 180-190 million tonnes of whey are generated annually (Guerrero-Rodríguez *et al*., 2012; Mieles-Cedeño *et al*., 2018). In Mexico, more than 2.4 million tonnes of whey are generated annually, but only 50% is processed (Mazorra-Manzano and Moreno-Hernández, 2019), the rest is discarded into water bodies or in the soil and causes serious environmental damage (Dainka *et al*., 2019).

González (2012) reports a biological oxygen demand (BOD) of 40 g L^{-1} and a chemical oxygen demand (COD) of 60 g L^{-1} with a biodegradability index (BOD5/COD) between 0.4 and 0.8, which exceed the limits allowed by the Official Mexican Standard NOM-001-ECOL-1996, on the maximum permissible limits of pollutants in discharges of wastewater for agricultural use, which is 0.2 g L^{-1} (DOF, 1997). Ramírez (2012) equates the polluting force of 5 L of whey to that of sewage produced in one day by a person.

To take advantage of whey lactose, proteins, and microelements (Chandrapala *et al*., 2015; Andrade *et al*., 2017), several studies have been carried out to apply it in human and animal food, production of bioplastics and fertilizers (Quille *et al*., 2021), and for agricultural irrigation (Araújo *et al*., 2017; Krause *et al*., 2017; Araújo *et al*., 2020). Where cheese factories with low technification predominate (Faría *et al*., 2002; Villegas-Soto *et al*., 2018), the use of sweet whey in agricultural applications represents a good option.

When whey is dumped into agricultural soil, it physically and chemically affects its structure (Carvalho *et al*., 2013) and therefore decreases agricultural yield (Parra, 2009; Araújo *et al*., 2013). However, other studies have shown that it improves soil aggregation (Kelling, 1981) and soil fertility (Robbins and Lehrsch, 1998; Jones *et al*., 1993).

Bioassays performed on BOD and COD are used to estimate the toxicity or potential biological impact that exposure to industrial effluents has on the physiological function of living organisms (Schultz *et al*., 2002). These tests determined the concentration that inhibits the germination of 50% of the exposed organisms, which is known as the mean lethal concentration (LC_{50}), as well as the concentration at which a sublethal effect (SLC) is produced, characterized by growth retardation (Uc-Peraza and Delgado-Blas, 2012).

In addition to tests carried out *in vitro*, Teac# and Bod#rl#u (2008) propose bioassays for phytotoxicity in hydroponic systems. Therefore, the objective was to evaluate the toxicity of different concentrations of whey in the irrigation of alfalfa (*Medicago sativa* L.) and corn (*Zea mays* L.) in the stages of germination and development of seedlings.

Materials and methods

The experiments were conducted in the Laboratory of Microbial Molecular Ecology of the Microbiological Sciences Research Center (CICM, for its acronym in Spanish) of the Meritorious Autonomous University of Puebla (ICUAP-BUAP, for its acronym in Spanish). Preparation and analysis of whey. Sweet whey was obtained from the production of fresh cheese in the cheese factory 'Lácteos Galeazzi' in Chipilo Puebla, which is characterized by its low acidity, neutral pH, and absence of salts. It was immediately refrigerated at 4 °C in 1 L Schott bottles until use. Its characterization was carried out by measuring, in triplicate, its pH with a Hanna 2010 potentiometer, and its acidity by titration expressed in degrees Dornic (°D) (AOAC, 1995).

Biological material

Unsterilized seeds of alfalfa (*Medicago sativa* L.) and corn (*Zea mays* L.) from local producers were used, which were selected according to shape and size, and stored in airtight amber bottles at room temperature. Experimental design, treatments, and variables. A completely randomized experimental design (CRED) was used.

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The concentrations evaluated were six whey concentrations in a range between 0 and 100% to measure the variable of LC_{50}, (effluent concentration that causes 50% mortality of the population in five days), and six concentrations between 0 and 20% to evaluate SLC (significant decrease in sprout and seedling development measured by total biomass, total length, and hypocotyl) in four acute and chronic toxicity bioassays (Eaton *et al*., 1995) with 3-10 repetitions with two plant species in two phenological stages as described in (Table 1).

Table 1 Experimental design.

Variable	Time (days)	Species	Phenological stage	Level of experimentation	Treatments (concentration % whey)
LC50	5	Alfalfa	Seed germination	*in vitro* *	0, 20, 40, 60, 80 ,100
LC50	5	Corn	Seed germination	*in vitro* *	0, 20, 40, 60, 80 ,100
SLC	10	Alfalfa	Sprout development	*in vitro*	0, 4, 8, 12, 16, 20
SLC	10	Corn	Sprout development	*in vitro*	0, 4, 8, 12, 16, 20
LC50	60	Alfalfa	Seedling emergence	hydroponic**	0, 20, 40, 60, 80 ,100
LC50	20	Corn	Seedling emergence	hydroponic	0, 20, 40, 60, 80 ,100
SLC	60	Alfalfa	Seedling development	hydroponic	0, 4, 8, 12, 16, 20
SLC	20	Corn	Seedling development	hydroponic	0, 4, 8, 12, 16, 20

* = in Petri dishes, darkness, and controlled temperature. ** = in seedling trays and Falcon tubes, sterile vermiculite, and controlled temperature-photoperiod conditions.

Description of experiments

The determination of LC_{50} was made with the objective of determining the concentration at which a residue produces 50% and more of the death of the organisms to which it is exposed and that is considered as potentially toxic. To calculate the LC_{50} of whey in alfalfa and corn seeds, Petri dishes were prepared with Whatman paper saturated with 5 ml of each dilution. Ten seeds were placed per box and per dilution, separating them to the maximum to allow their development (three boxes per dilution). Each box was sealed with Parafilm®, wrapped in black plastic, and incubated at 20±2°C for 5 days (Wang, 1987; Navarro *et al*., 2006).

The percentage of germinated seeds was plotted against the concentration of whey and the value that produced the inhibition of germination in 50% of the population was obtained by extrapolation, as well as by the equation obtained from the polynomial regression. Those that did not germinate after 20 days, even irrigated with drinking water, were considered dead. The experiment was done three times, with whey of different batches and dates and the results of the most illustrative experiment were reported.

The objective of determining the sublethal concentration (SLC) is to know if a residue, although not toxic, affects the growth in the early stages of an organism. The SLC of sweet whey was determined at 10 days in dilutions less than 20%, in which the decrease in the development of sprouts (length of the hypocotyl, length, and total weight) was evaluated, under the same conditions of the previous experiment according to the method described by Tiquia and

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Tam (2000). To evaluate the LC_{50} of sweet whey in alfalfa and corn seedlings, an expanded polystyrene seedling tray was prepared with sterile vermiculite as a substrate, with 10 alfalfa seeds per well, and then five of them were assigned for each treatment.

In parallel, 50 ml Falcon tubes were prepared with 30 ml of sterile vermiculite, and one corn seed per tube was sown with 10 repetitions per treatment. The material was placed under controlled incubation conditions (20 ±4 °C with photoperiod 12-12) in a TE-4002 Tecnal Trae® climatic chamber and irrigated with 5 ml of each treatment for 20 days with a serological pipette in the case of corn and 60 days for alfalfa with a hypodermic syringe. The number of seedlings that emerged was counted, the percentage of these was plotted against the concentration of whey, and the value that inhibited the emergence in 50% of the population was obtained by interpolation and by the equation obtained by the regression of the polynomial curve.

To determine the SLC of sweet whey in alfalfa and corn seedlings, dilutions of less than 20%were tested. The decrease in development (biomass expressed in dry weight, total length, and root length with respect to the drinking water control) was evaluated according to the method described by Tiquia and Tam (2000) at 20 days for corn and 60 for alfalfa, irrigation with treatments began once the seedlings emerged and they were irrigated at their base. It was considered that there was a significant sublethal effect when the comparison of means between each treatment against the control was statistically significantly lower according to the t-Student test ($p< 0.05$).

For the determination of the length of sprouts and seedlings, an LCD Metric® digital electronic vernier with a maximum capacity of 150 mm was used. Biomass determinations were made by gravimetry at constant weight; that is, it was verified that there was no variation in the reading by successive exposure to drying in a convection oven in an Ohaus Adventurer® analytical balance with a maximum capacity of 420 g and resolution of 0.001 g. Drying was done on a Thermo Scientific® convection oven equipped with a thermometer to adjust the drying temperature.

Statistical analysis. To keep the coefficient of variation below 30%, a pair of data (maximum and minimum) was eliminated when necessary. The statistical packages used were Excel and SAS v 9.4 (2004).

Results and discussion

Physicochemical analysis. Two types of whey were found as a by-product of cheese making: sweet and salty. The first, which comes mainly from the production of fresh cheese, presented a pH close to neutral (6.8 ±0.3) and low acidity (19 ±0.5 °D) because it had no added salts and had low concentrations of calcium chloride. The second, which comes from the production of quesillo, cottage cheese, and ranch cheese, had a pH of 5.41 ±0.3 and acidity of 38 ±0.6 °Dornic. These results are similar to those reported by Panesar *et al*. (2010) for sweet whey and by Guerrero-Rodríguez *et al*. (2012) for acid whey.

It is important to consider that the characteristics of whey vary depending on the type of cheese that is processed and that its agricultural use will depend mainly on whether it is free of added salt. On the other hand, whey, although it has a low acidity at the time it is generated, tends to acidify rapidly naturally due to the development of its microbial flora: the lactic acid generated in this process can have a beneficial effect in agricultural processes such as in the chelation and solubilization of phosphates (Paredes-Mendoza and Espinosa-Victoria, 2010) but it will be a priority to take care of the development of pathogenic microorganisms with controlled fermentation processes, for example with the technology of beneficial and/or efficient microorganisms (Morocho and Leiva, 2019).

LC_{50} in seed germination. At concentrations of 0 to 40% whey, germination is maintained at almost 100%, however, with an LC_{50}-5, germination fell to 53% in alfalfa seeds (Figure 1), a value obtained graphically by interpolation, and that coincides when substituting the value y= 50 (50%of the population that does not survive) in the polynomial equation. The decrease in germination

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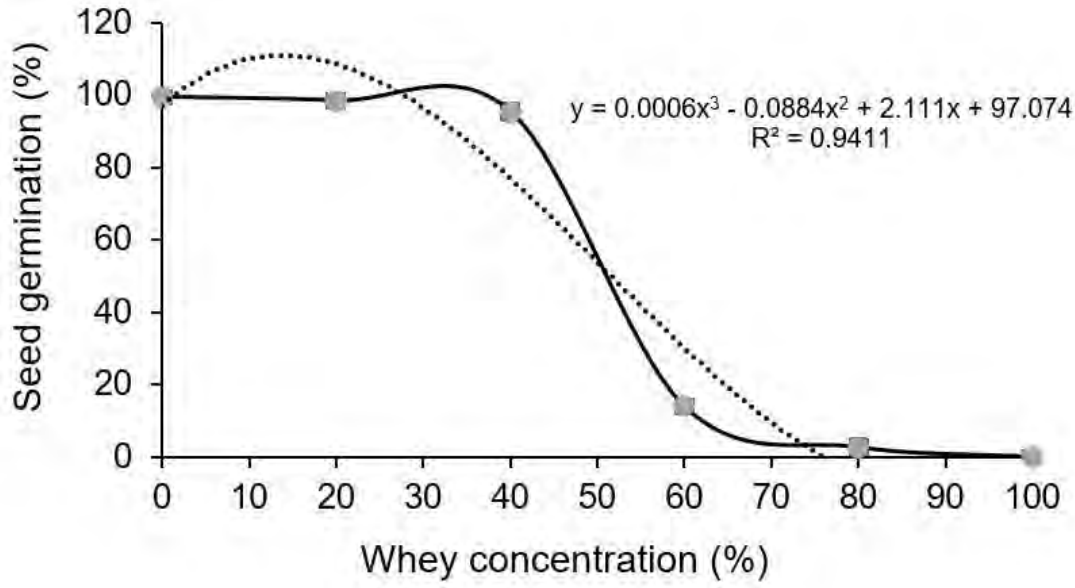
from 40% whey concentration is due to the high concentration of NaCl, which raises osmotic pressure and caused an adverse physiological effect.

Figure 1. Toxic effect of sweet whey at different concentrations on the germination of alfalfa seeds at five days. Corrected values that were considered with 83.5% germination of the control.



Dantas *et al*. (2005) found similar results in germination of alfalfa seeds exposed to salt stress. Laynez-Garsaball *et al*. (2008) observed a decrease in germination due to the increase in osmotic potential exerted by NaCl, while Porta *et al*. (1999) demonstrated that the total inhibition of germination in high salt concentrations is due to the accumulation of chloride, which decreases water absorption and affects the germination rate.

For their part, Mahdavi and Sanavy (2007) report that ions present in whey can also cause hardening of cell walls and affect seedling growth. LC_{50} was not obtained in corn, because in all treatments there was contamination by filamentous fungi. This may be due to the natural microbial load of corn, which is favored by the contribution of nutrients from whey.

SLC in sprout development. Sweet whey showed a sublethal effect at concentrations greater than 12% in the development of alfalfa sprouts at 10 days of exposure, observing significant differences between the total length of sprouts and hypocotyls compared to the drinking water control ($p< 0.05$) (Figure 2 a and b), although no effects were observed on total biomass (data not presented). In similar works, Navarro *et al*. (2006) report the inhibition in the growth of hypocotyls of chicory, lettuce, and endive seeds exposed to neutralized effluents from citrus and wine industries diluted to 10^3.

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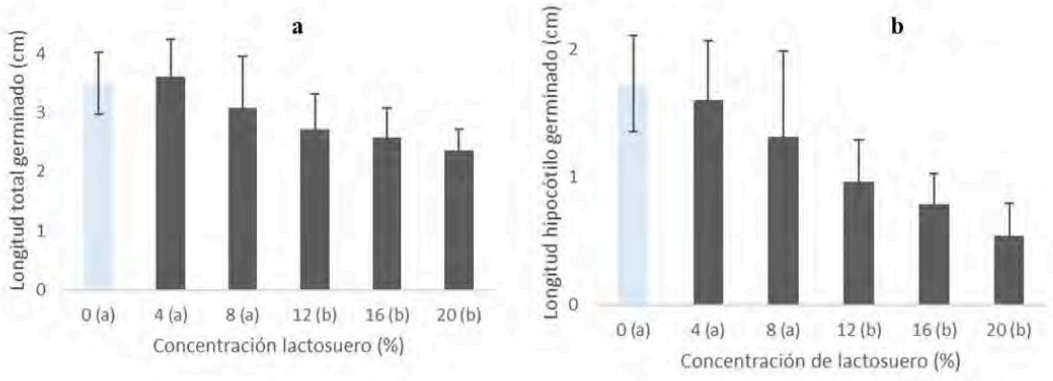
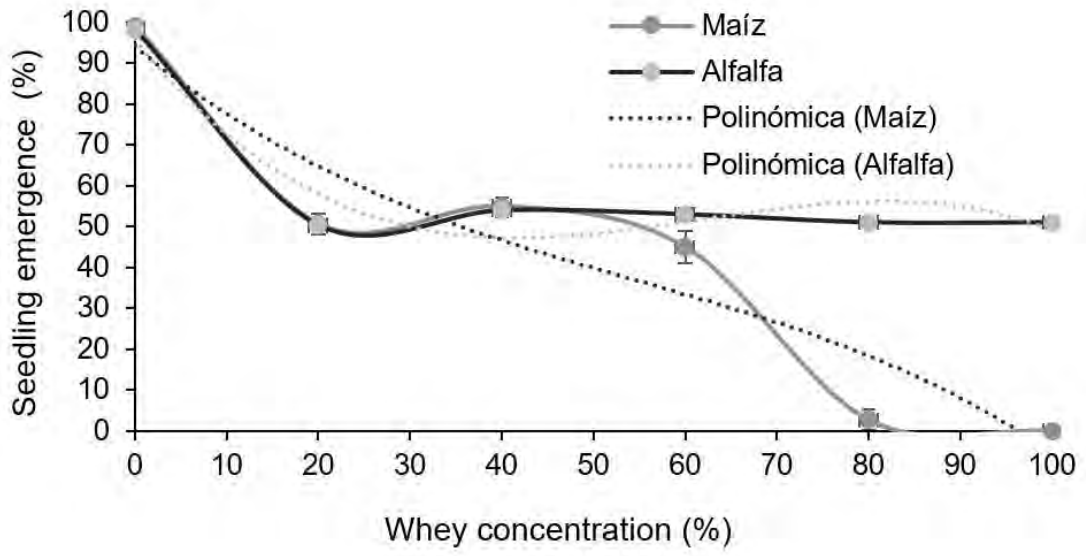
Figure 2. Sublethal effect of sweet whey on total length (a) and length of the hypocotyl; and (b) 10-day-old sprouts of alfalfa. Different letters between columns indicate a significant difference (t-Student $p< 0.05$).



If this value is compared with that obtained in the present study, in which inhibition of hypocotyl development was observed at a concentration of 12% (10^1), then it can be affirmed that sweet whey has low toxicity. On the other hand, SLC values were not obtained for corn because, as in the previous experiment, the seeds were contaminated with filamentous fungi in all treatments.

LC_{50} in seedling emergence. The lethal concentration of sweet whey in corn seedlings was obtained at a concentration of 40%, and similarly in alfalfa seedlings, although 100% lethality was not observed in alfalfa at high concentrations of whey, the survival of 50% of the population is evidence that this species is more tolerant to sweet whey than corn (Figure 3). Various authors such as Wang (1987); Navarro *et al*. (2006), in works carried out on lettuce, found differences in tolerance to the toxicity of different substances in different species, with lettuce being one of the most used species in this type of study due to its high sensitivity.

Figure 3. Toxic effect of sweet whey at different concentrations on the emergence of seedlings of corn (*Zea mays* L.) and alfalfa (*Medicago sativa* L.).



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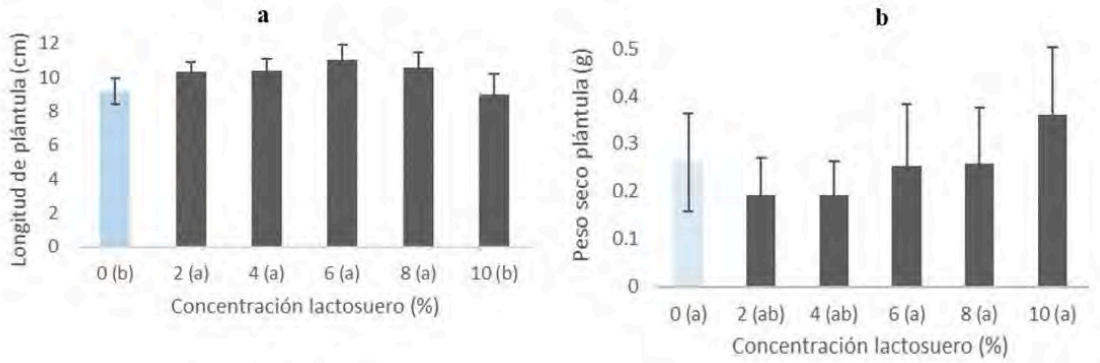
Alfalfa tolerance can be explained because it is a seed more resistant to low pH, as reported by Köpp *et al*. (2011), who found that alfalfa can grow optimally between pH 5 and 6 but can tolerate even lower pH values, while the optimal pH of corn growth is 6 to 7.2 and exhibits growth inhibition at pH values lower than 5 (Aldrich and Long, 1994).

SLC in seedling development. No differences were observed in variations in total length in all concentrations studied ($p< 0.5$) so it is assumed that sweet whey has no sublethal effect at a concentration lower than 10%, in corn or alfalfa seedlings; however, a beneficial effect was observed at a concentration of 5 ±2.6% for alfalfa length ($p< 0.05$) (Figure 4a and 4b).

Figure 4. Sublethal effect of sweet whey on length (a) and biomass; and (b) of 60-day-old alfalfa seedlings. Different letters between columns indicate a significant difference (t-Student $p< 0.05$).



The differences observed between experiments with seeds and seedlings indicate that the phytotoxicity is lower for alfalfa than for corn and that in both species, whey is more toxic in the early stages of development. On the other hand, it is necessary to carry out more research to evaluate what is the optimal concentration in other stages of the development of the plants studied, as well as the use of whey under field conditions.

Conclusions

According to the chemical characterization carried out, it was found that the main wheys generated in Chipilo Puebla are sweet and acid-salty. The first is the one with the greatest potential for biotechnological use since it has low toxicity (LC_{50} of 55%), although it was determined that at a concentration of 12%, it has a negative effect on the growth of 10-day-old alfalfa sprouts, which indicates that sweet whey has low toxicity, with high probabilities to be used safely in the agricultural irrigation of species such as alfalfa and corn.

Its use is recommended at concentrations lower than 12% in order to maximize its use and at concentrations less than 4% as a plant growth promoter. The use of agro-industrial by-products represents an area of research with great potential.

The use of whey to make products of commercial interest, particularly as a raw material to produce biofuels, metabolites, and biofertilizers by biotechnological transformation, is gaining increasing attention. It is suggested to continue studying its use as a nutrient medium to promote the growth of microorganisms of agricultural interest such as fungi and bacteria that fix nitrogen, solubilize phosphorus, and are antagonistic to phytopathogens and compare its effect on corn and alfalfa compared to untreated whey.

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