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MICROENCAPSULATION OF THE ALLELOCHEMICAL COMPOUNDS AND STUDY OF THEIR RELEASE FROM DIFFERENT PRODUCTS

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This research aims to use extracts of tropane alkaloids in *Datura stramonium* using known extraction procedures followed by microencapsulation in order to facilitate environmental protection and sustainable development. The advantage lies in the fact that the release of extracts of plants can be controlled and extended by the chemical mediation. The microcapsules were prepared by the process of the interfacial cross–linking. Tropane alkaloids inhibit the proteinases and native/active site shall be secured, by introducing the microcapsules incorporated in the aqueous phase. The paper studied the effects of the influence of pH of the aqueous phase, PMMA, terephthaloyl chloride concentrations, and the rate of stirring on the morphology and size of the microcapsules. The pH was a determining factor in the amount of microcapsules, influencing the encapsulating yield. The size of the microcapsules varied depending on the speed of mixing 50, 100 and 150 rpm respectively. Infrared spectroscopy was performed on the microcapsules prepared under different conditions. Between the spectral changes, the morphology and size of microcapsules may be set a correlation. It was found that the active substances (tropane alkaloids) protected by encapsulation kept constant their activity after microencapsulation.

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1. Introduction

The current trend in the chemistry of allelochemical compounds and biocides is the knowledge of transformations that take place and implementation of legislation regarding the marketing of these products. Synergistic allelochemical and biocidal products open up the way to utilization of new materials with important practical applications. Secondary metabolites are biosynthesized by plants and may have different functions (growth inhibitors, natural growth hormones, etc.) [1]. Allelopathic compounds have a role in determining plant diversity, dominance and success, as well as in the development of natural vegetation [2] and plant productivity. Indiscriminate use of synthetic herbicides is determining threats to ecosystems through imbalance of soil microorganisms, deficiency of nutrients and physico–chemical changes that result in decreased crop productivity [3]. The use of allelochemical compounds and biocides in the agricultural management can reduce the amount of synthetic herbicides, fungicides, and insecticides, reducing the negative effects and damage to the environment. *Datura stramonium*,

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commonly known as jimson weed, presents a rich alkaloid spectrum. Along with the scopolamine, which is the main alkaloid, it contains hyoscyamine, and teloidine. From *D. stramonium* plants were extracted only scopolamine and atropine, because by extraction the hyoscyamine (the tropine ester with the tropic acid (–), is a left–handed), switch to atropine (the tropin ester with tropic acid (+), a racemate) [4]. Microencapsulation is a process by which small drops or particles of liquid or solid material are surrounded and coated with a continuous layer of polymeric material [5]. Micro–encapsulation is a process of coating an active substance (molecules, solid or liquid corpuscles formed by globules of different materials) in order to achieve micrometer–sized corpuscles. The results of microencapsulation are microparticles, microcapsules (MICs) or microspheres (MISs), depending on the particularities of the system reported to the internal morphology and structure (are smaller than 1mm) [6]. The morphology of the corpuscles depends on the core material and exterior material deposition process. Mononuclear microcapsules (core–shell) comprise a shell around the active substance, while the polynuclear capsules present a multi–core contained in an outer material. In the case of encapsulation, the core material is homogeneously distributed in an exterior material [7]. Given the practical aspects, common classification of the process includes: matrixencapsulation and Core–Shell–Encapsulation [8]. MIS are spherical particles of micrometer size and (1–1000 μm). The term microspheres are used for particle sizes ranging from 0.02 to 2000 μm [9]. MICs are microcapsules composed of one or more active ingredients (core: solid, liquid, and gas) and a protective matrix (wall / cover). Active material can be in the form of a central core surrounded by inert polymer ((MIC–type reservoir or mononuclear) or dispersed network in a polymeric matrix (MIC–type monolithic) [10]. The allelochemical compounds and biocides are active substances that destroy and prevent pests or microorganisms infestation [11]. These are substances used against living body [12] to prevent or destroy the growth of microorganisms [13] or for weed control in organic farming. The use allelochemical compounds and biocides help to protect the uncontrolled use of xenobiotics in the environment [14].

The main objective of this paper is to investigate the use of some products with controlled release of allelochemical and biocides substances (tropane alkaloids), with the aim of prolonging their action and for the environmental protection. A preparation based on alkaloids (atropine and scopolamine) was tested for potential biocidal action, for weeds control, being biodegradable, having low toxicity to humans and animals and with low environmental impact. This product is based on chemical compounds from natural plant extracts, which are designed to reduce or eliminate pollution.

2. Materials and methods

2.1. Plant material

D. stramonium plants were collected from the Banat area (western Romania) in September 2011, at full flowering stage, when the fruit is matured and seed dispersal took place.

Chemicals. Scopolamine, atropine, acetonitrile and methanol for HPLC were purchased from Sigma–Aldrich, and double distilled water was prepared in house.

Tropane alkaloids extraction. The powdered plant material (0.5 g), obtained from *D. stramonium* was extracted three times for 30 min with 15 mL of chloroform, methanol and 25% ammonia 15:15:01 (v/v/v) with an ultrasonic device. It was kept at room temperature for 1 hour, filtered and washed twice with 1 mL $CHCl_3$, the solvent was evaporated to dryness. The residue was dissolved in 5 mL of $CHCl_3$ and 2 mL of H_2SO_4 1N. The organic fraction of $CHCl_3$ was removed. The aqueous solution was reduced to basic (pH 10) with 25% ammonium hydroxide, on ice. Alkaloids were extracted with 2 mL of $CHCl_3$ and 1 mL of chloroform. After the addition of anhydrous Na_2SO_4, the solution was filtered, and the residue was washed with 1–2 mL of $CHCl_3$. The solvent was evaporated to dryness under reduced pressure and the residue (total extract) was dissolved in the appropriate volume (1–2 ml) of methanol [15].

*Reference tropane alkaloids: S*tandard solution of scopolamine and atropine (0,01 g) was prepared in 100 mL metanol.

High performance liquid chromatography (HPLC). Scopolamine and atropine were obtained from the extract of *D. stramonium*. The alkaloids were highlighted by characteristic reactions and identified with HPLC–UV method for the simultaneous determination of scopolamine and atropine from an aqueous extract. For quantitative determination of both substances the method used was ionic pairs and DAD. The HPLC method used a Kromasil 100–5C8 column (250x4.6mm) with gradient elution and the temperature 25°C. Mobile phase: acetonitrile 25% and 75% aqueous solution (5 mM sodium 1–heptanesulphonate monohydrate, pH 3.5). The detection of the analytes was performed in UV at $\lambda = 230 \pm 4$nm with reference $\lambda = 360 \pm 8$ nm. The calibration curve is linear in the range from 0.13 to 13.75 μg/mL (r=0.9951, n=8) for scopolamine and 0.25 to 25.5 μg/mL (r=0.9999, n=8), respectively, for atropine. Data were generated by ChromeGate using atropine and (–) scopolamine as standard samples [16-17].

Quantification of the tropane alkaloids. The quantitative determination was carried out by external standard method. Standard solutions of atropine and scopolamine (4, 10, 25, 50, 100, 200, 400 ppm) were prepared in methanol. 20 μL of each standard was injected on the HPLC column [18].

2.2. Microencapsulation process. The microcapsules were prepared by modification of the reticular surface (technique for polysaccharides). Afterwards, the microcapsules were resuspended in water and then lyophilized. The variances were in the rate of stirring and the composition of the aqueous phase in the preparation of microcapsules. Phosphate buffer solution (0.1 M) was used at pH 5.0, 6.0, and 7.0 and 0.5 m carbonate buffer, at pH 8.0 and 9.8. PMMA and starch concentrations were 10 and 0–5%. Terephthaloyl chloride concentrations were 2, 3, and 5%. The series were prepared three times.

2.2.1. Preparation of microcapsules. Tropane alkaloids and microparticles were prepared by solvent evaporation technique. An amount of polymer was added to acetone to obtain a homogeneous solution. A quantity of alkaloids was dispersed in this solution by continuous mixing until it was obtained dispersion. When it reached room temperature, the acetone evaporated and fine, settleable particulates were obtained [19]. The microparticles were washed with n–hexane and water, dried at room temperature, and then passed through the sieve. Allelochemical substance preparations were stored in glass containers in the absence of air. Three series were prepared with different proportions on the basis of the materials listed (alkaloids: polymer = 1:1, 1:1.5 and 1:2 (g / g), noted as microparticles P_1, P_2 and P_3, and tablets C_1, C_2 and C_3).

2.2.2. Tropane alkaloids of microcapsules loading. 25 mg of preparation with allelochemical substances were added to 100 mL of phosphate buffer to get pH =6.8 and subjected sonication for three hours. Then the solution was filtered, appropriate dilutions were made from filtered extract and analyzed by absorption spectroscopy at 272 nm UV–VIS Spectrophotometer (T60U, PG Instruments Limited, UV ® WIN version 5.05); quartz cuvettes of 1 cm).

2.2.3. Preparation of tropane alkaloids (atropine and scopolamine) tablets. Allelochemical substance preparations were mixed with magnesium stearate (1%) and talc (0.5%) and compressed into tablets in order to study their dissolution behavior.

2.2.4. Physical tests of tablets with tropane alkaloids. USP criteria mentioned in Disolution and Drug Released describe a range of 3: to 24 to meet individual tests requests of Pharmacopoeia. The automatic dissolution tester has a great working efficiency and some advantages such as improved methodology, work accuracy and reproducibility. The cylinder (25 rpm) for friability has an internal diameter of 287 mm and a depth of 38 mm

2.2.5. Dissolution studies (influence of pH, the rate of stirring and storage conditions for the rate of release of alkaloids). Dissolution studies were performed in 900 mL of distilled water, with the temperature maintained at 37.0 ± 0.5 °C and stirred at 50 rpm. Samples (5 mL) were filtered, collected at the times established and analyzed by UV absorption spectroscopy at 272 nm. The dissolution test was performed three times. Tablet formulation (C_2) was tested at different values of pH 1.0 (0.1 M HCl), distilled water and pH 6.8 (phosphate buffer). Allelochemical substances preparation was tested at stirring speeds 50, 100 and 150 rpm; it was packed in airtight glass containers and stored at 25 °C/40 °C. Checking the stability was performed by dissolution tests after one, two and three months. The tests were conducted three times.

2.2.6. Analysis of tropane alkaloids of microcapsules release data. In order to study the kinetics of release, the data were represented in the kinetic model: zero–order, first–order and Higuchi model.

$$\log F=\mathrm{Log}F_0-k_1\cdot t/2.303,\ F=K_h\cdot t^{1/2}$$

where K0 and K1 are kinetics constants of zero–order and first order. Kh–constant reflects the variables of the system and t is time in hours. Hixson–Crowell law was used to evaluate the release of alkaloids, surface modification and diameter of particle/tablets: $F0_{1/3}–Ft_{1/3} = Khc\cdot t$, where–Ft is the amount of alkaloid at time t, F0, the initial amount of alkaloid in the tablet and Khc–constant Hixson–Crowell rate equation $F = K \cdot t$. K–constant rate equation Korsmeyer, n–exponent for the alkaloids release mechanism and F–fraction of alkaloids and alkaloid dissolved at time t.

2.2.7. Coefficient of determination (r^2). The similarity factor (C_2) was used to compare the dissolution profiles.

$$C_2=50\cdot\log[1+1/n\cdot\sum(R_t-T_t)^2]^{-0.5}\cdot 100$$

n–points of dissolution and Rt, Tt dissolution profiles at the time t, for the same reference point and time and for testing profiles at dissolution. The value of C_2 with values between 50 and 100 indicates that the two dissolution profiles are similar.

2.2.8 Microencapsulation tropane alkaloids efficiency (MEE). The efficiency of microencapsulation was calculated according to:

MEE=(total *alkaloids*–extractable *alkaloids*) 100/total *alkaloids*

Extractable alkaloids were measured after a slow shaking. MEE was used as a factor in assessing the quality of the preparation coating with allelochemical substances and defined as part of the alkaloids, from HS microcapsules, that have not been removed by a solvent or environment exposed.

2.2.9. Microencapsulation tropane alkaloids yield (MET). Microencapsulation yield is defined as the ratio of the core material in the final dry preparations containg allelochemical substance in the emulsion, and is calculated as follows:

MET=core material in microcapsulea (g/100g solid)/ core material in emulsion (g/100 g solid)

2.2.10. Stabilization tropane alkaloids of microcapsules factor. The effectiveness of the NPEs tests and their mixtures, as an antioxidant, was expressed as stabilization factor as follows:

F=(induction period with inhibitor)100/induction period without inhibitor

2.2.11. Infrared spectra. IR spectra study samples were prepared according to standard techniques: 10 mg preparations with allelochemical substances, were lyophilized, and grounded with 200 mg of KBr. The mixture was compressed into tablets, 1 mm thickness under a pressure of 10 kPa. The spectra were obtained with *JASCO 660 PLUS* spectrophotometer in the optical area from 4000 cm^{-1} to 400 cm^{-1}.

2.2.12. Statistical analysis. All experiments were performed in three series, and the differences between experiments were analyzed by Student's t–test using Microsoft Excel 2010.

3. Results and discussion

Datura stramonium alkaloids distribution. The results of quantitative analysis of the main tropane alkaloids (atropine and scopolamine) of *D. stramonium* indicated a greater amount of atropine than scopolamine in all samples studied (Fig. 1 and Table 1).

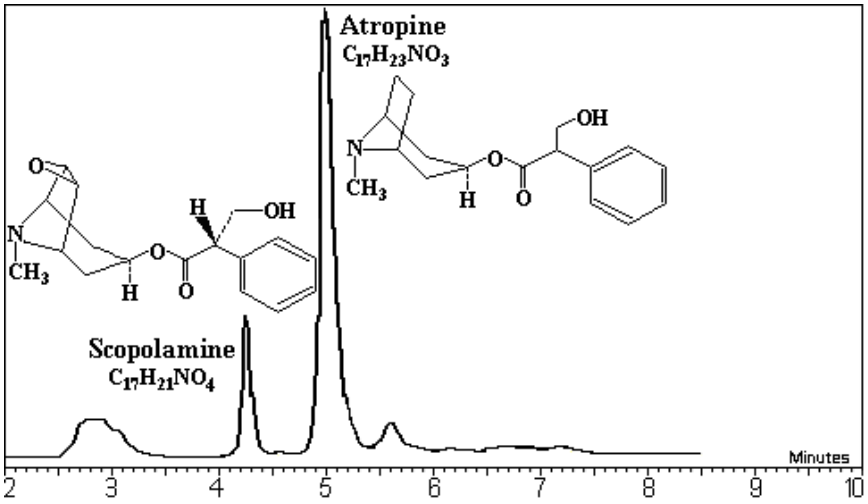

Fig. 1. HPLC chromatogram of Datura stramonium.

Linear regression equation and correlation coefficient for atropine and scopolamine was determined, and the result are presented in Table 1.

Table 1. Linear regression equation and correlation coefficient for atropine and scopolamine.

No.	R^2	**Linear regression equation y=ax+b**	**Compound**
1	0.9989	3871.7 X +1527.2	Atropine
2	0.9987	1737.2 X +9707.9	Scopolamine

From the micro–tablets of alkaloids PMMA (100 mg) were prepared compressed microparticles by the solvent evaporation method and were characterized by dissolution.

Stability studies of tropane alkaloids. PMMA has no effect of aging during storage at different temperatures. Were studied the changes of allelochemical substances formulations release stored at room temperature, 25 and 40°C. But there was a difference in the release profiles of the alkaloid tablets after storage for 3 months at 40°C, compared with the initial release profile. The release rate of the alkaloids was reduced to 40°C reported to the initial value [20].

In the release profile of the alkaloids was not observed any visible change during the 3 months.

Poly (methyl methacrylate), a copolymer of anionic methacrylic acid and methacrylic has free carboxyl groups, giving its sensitive pH. Poly (methyl methacrylate) is insoluble below pH 7 and was used to encapsulate the tropane alkaloids by solvent evaporation method.

Preparations with allelochemical substances will slow the release of tropane alkaloids in acid pH of soil. Increasing the ratio of poly (methyl methacrylate) in the preparations will reduce the diffusion of water molecules within the polymer, thereby reducing the degree of swelling of the microcapsules, resulting in slow release of tropane alkaloids [21].

The presence of high percentage of PMMA in preparations with allelochemical substances gave a hydrophobic character. At a high ratio of polymer, the capsules are becoming sealed in the dissolution medium and the effect will be slow release effect of tropane alkaloids, P_3 formulation.

Table 2. Characteristics of tropane alkaloids of microcapsules mixed with distinct compositions (atropine and scopolamine)–poly(methyl methacrylate)

Measured factors	Microcapsules		
	1:1 (w/w)	1:1.5 (w/w)	1:2 (w/w)
Average particle size (lm)	162.03	79.35	65.21
Moisture (g/100 g)	5.23	5.68	5.64

Water activity (aw)	0.292	0.278	0.262
MEY (%)	94.62	94.20	94.25
MEE (%)	45.08	49.62	89.02

The information indicates that the reduction of tropane alkaloids from the preparations with allelochemical substances may be due to the decrease of the diffusion in the dissolution medium inside microcapsules (PMMA hydrophobicity in water).

Table 3. Induction time and stabilization factor of distinct tropane alkaloids of microcapsule–poly(methyl methacrylate)

Microcapsules	Induction time (h)	Stabilization factor
1:1 (w/w)	80	106.07
1:1.5 (w/w)	23.50	113.76
1:2 (w/w)	23.95	115.57

The size of the allelochemical substance preparations ranged from 165–180 μm.

*In vitro tropane alkaloids of microcapsule release profiles. S*tudies have shown that increasing the amount of PMMA on *in vitro* release of tropane alkaloids depends on different formulations [22]. At low concentrations of PMMA (P_1), 80% of the tropane alkaloids were released in 180 minutes while 63% and 33% of the tropane alkaloids were released from the formula which had large amounts of PMMA (P_2 and P_3) at the same time.

Evaluation of the release kinetics of the tropane alkaloids from the microcapsule. It was noted that information could better correspond to Higuchi model. Higuchi model R^2 values were found to be higher compared to other kinetic values.

Influence of pH on tropane alkaloids release of test tablets. C_2 was selected as preparation with allelochemical substances and further evaluated (it indicates best the release profile of the alkaloids from the preparation at different values of pH, 1.0 and respectively 6.8). Differences were observed in the release characteristics at both pH values.

Table 4. Values of release rate constant k, correlation coefficient R^2 /$T_{70\%}$ obtained from data of tropane alkaloids tablets (C_1, C_2 and C_3) containing various drug to polymer ratios at distinct pH and stirring speed

Formulations with their dissolution conditions	K	R^2	$T_{70\%}$
C_1 in distilled water at 50 rpm	10.604	0.9871	7.9878
C_2 in distilled water at 50 rpm	8.5057	0.9775	9.7887
C_3 in distilled water at 50 rpm	7.4022	0.7766	13.2873
C_2 in pH 1.0 at 50 rpm	5.8816	0.7581	14.8422
C_2 in pH 6.8 at 50 rpm	8.0873	0.9613	10.6912
C_2 in distilled water at 100 rpm	8.4986	0.9627	10.3897
C_2 in distilled water at 150 rpm	9.0894	0.9756	10.0316

*Influence of stirring speed on tropane alkaloids release of tablets s*hows the release profile of the preparations with allelochemical substances at three different stirring speeds. When the mixing speed is increased, the disintegration of the formulations with allelochemical substances (tropane alkaloids) was not affected by erosion, indicating a similarity of micro–encapsulation system.

There were no major changes in release of the preparations with allelochemical substances at the three stirring speeds [23]. Table 5 presents the C_2 values obtained from *in vitro* release mode of tropane alkaloids micro tablets compared at 100 rpm versus 150 rpm and respectively at 100 rpm vs. 50 rpm.

Table 5. C_2–values determined from tropane alkaloids of microcapsule release data of test tablets (C_2) at distinct pH and stirring speeds (rpm).

Comparison	C_2 –value
Distilled water / pH 6.0	29.05

Distilled water /pH 8.2	53.24
Distilled water /pH 9.8	58.12
100 rpm / 150 rpm	61.24
100 rpm /50 rpm	57.42
50 rpm /150 rpm	57.52

The values obtained in test C_2 for 100 rpm and 150 rpm, respectively, was greater than 50, and the dissolution data were similar due to erosion and disintegration of the matrix at a high speed

C_2 value at 100 rpm and 50 rpm was less than at 50 and the dissolution data could not be counterbalanced due to the gradual swelling of the matrix. T70% was calculated in Table 4 but it does not present significant differences between the three values of stirring speed.

The structure of tropane alkaloids according to IR–spectroscopy.

The IR spectra of the formulations with allelochemical substances (tropane alkaloids) 0.4%, by interfacial cross–linking of the various pH values is shown in Table 6. The absorption bands from 3600–3200 cm^{-1} determined by the vibrations of the O–H polysaccharides links were not taken into consideration.

Table 6. Typical infrared absorption peak areas for specific regions

Group frequency, wavenumber (cm^{-1})/ Assignment	**Functional Class**
1000–1100 cm^{-1} [*1050–1000 Aliphatic phosphates (P–O–C stretch)*]	mono–, oligo– carbohydrates
1100–1200 cm^{-1} [*1200–1100 Tertiary amine, CN stretch*]	acid or ester
1300–1400 cm^{-1} [*1300–1200 Primary or secondary, OH, in–plane bend 1400–1300 Phenol or tertiary alcohol, OH bend*]	Amide, phenyl groups
1500–1600 cm^{-1} [*1600–1500/1400–1300 Carboxylate (carboxylic acid salt); 1690–1620 Amide*]	amino acids
1620–1750 cm^{-1} [*1620–1580 Aromatic ring stretch*] and 1100 – 1000 cm^{-1} *(carboxylic acid salt);*	Aldehydes, cetones, esters

The band at 1545 cm^{-1} was characteristic of the spectra of the preparations with allelochemical substances. The band at 1789 cm^{-1} was assigned to the asymmetric link C=O stretching vibration anhydride. The other change in the spectrum was observed at 1731 cm^{-1}, due to C=O stretching vibration of aromatic acid esters. The intensities of these peaks increase at increasing pH. The band at 1617 cm^{-1}, is corresponding to the protein content [24], by increasing the pH. In the IR spectra of the preparations with allelochemical substances changes have occurred in the region corresponding to deformation of the O–H bonds (1400–1200 cm^{-1}), C–O valence vibrations of esters (1300–1000 cm^{-1}) [25] and aromatic anhydrides (1290–1300 cm^{-1}) [26]. A significant increase in the higher area o the peaks, with amaximum at 1281 cm^{-1} is attributable to the formation of esters. Between 1200–1000 cm^{-1}, the spectra of the preparations with allelochemical substances, determine intense band due to vibrations by valence C–O from polysaccharide [27]. Absorption bands at 1100 and 1000 cm^{-1} are attributable to the ester group [28] involving the hydroxy groups and functional groups of various proteins involved in the polycondensation, due to the increase of pH. At low pH, it can be observed the bands determined by the preparations with allelochemical substances formed by acylating the–OH site of the protein amino groups and carboxyl groups. At increased pH, the acylation of carboxylic groups and of some aminoacid protein determines the occurrence of some bands (determined by the intermolecular bonds of proteins). The preparations with allelochemical substances treated with slightly alkaline buffer become more flexible (have fewer cross–links). The proteins in the structure of the preparations with allelochemical substances are less ordered at 1617 cm^{-1}. The

changes in the structure of the preparations with allelochemical substances affect the rate of degradation, at the three pH values characteristic of the soil solution.

4. Conclusion

For the release of tropane alkaloids from the preparations with allelochemical substances were used two different core materials with similar chemical properties but different physical characteristics.

Conclusions were supported by the investigations used to monitor both the absorption and the release, the influence of pH, stirring speed and storage conditions for the rate of release of alkaloids. In this paper it is shown that the active substances can be effectively encapsulated in a protective coating.

The main advantage is that by using preparations containing allelochimicals and biocidal substances, the active ingredient release can be controlled and that the mechanical properties of the soil may remain intact, even at high concentrations of tropane alkaloids.

D. stramonium plants are suitable to be used for situations where some weeds become harmful and, on the other hand, plant crops are not suitable crops for occasionally and/or transitional cultivation near the plants of *D. stramonium*.

Sustainable development of agriculture associated with the "green" revolution in the context of the Food Quality Protection Act, would be possible through the design and use of natural products for pest control. These new regulations should reduce the amount of available synthetic pesticides in agriculture.

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