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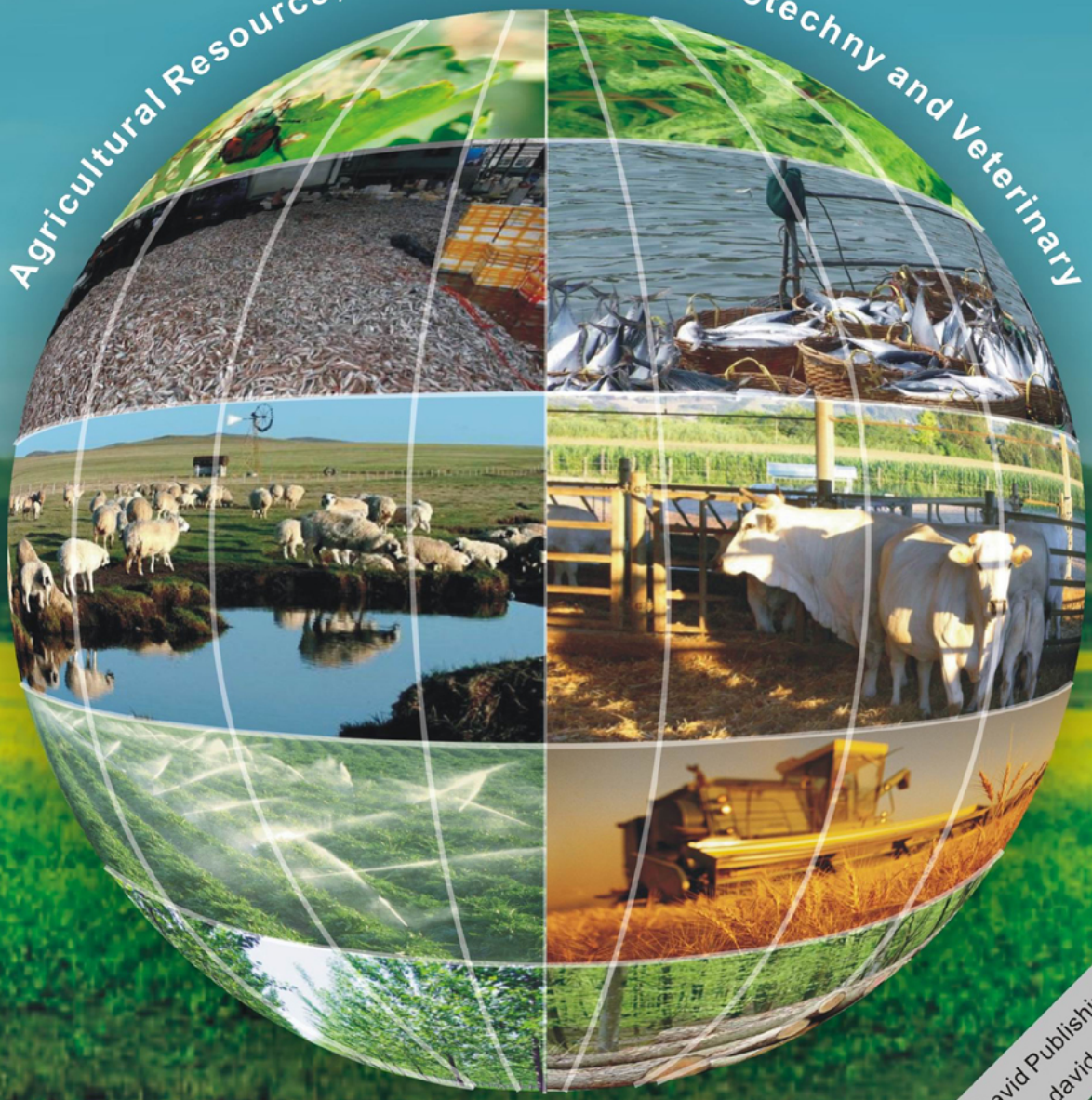

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Borderline Products between Bio-fertilizers/ Bio-effectors and Plant Protectants: The Role of Microbial Consortia

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Abstract: In the delicate normative balance, at European Union (EU) level of the borderline products (i.e., between plant protectants and bio-fertilizers/bio-effectors) containing microbial consortia (MC) instead of single microbial strains, the most relevant factors influencing the categorization of the products are the intention of use, the cell density and the mode of action. For the latter, the basic difference between the two types of products is that a plant protectant has a targeted activity on plant pathogens, while a bio-fertilizer acts indirectly by nourishing and fortifying the host plant (healthier plant), thus inducing a generalized resistance to the onset of pathological status, irrespective of its origin and nature. Case-studies are presented on the effectiveness of MC as bio-fertilizers/bio-effectors on different crops. Bio-fertilizers exhibit a double effect—biotic and abiotic, leading to the fortification of the crop plant linked to its more effective water and nutrient uptakes as well as to a generalized healthier status. This in turn leads to a higher resistance to diseases. In addition, bio-fertilizers play a relevant role on the reduction of environmental impacts due to chemical fertilizers, e.g., by facilitating the uptake of phosphorus (P), thus reducing the need of P fertilization. Although finding a scientifically-based balance between regulatory need and marketing constraint is not always an easy task, the availability of scientific advancements combined to common sense should help in describing positive effects and risk profiles of MC in agriculture.

Key words: Bio-fertilizers/bio-effectors, plant protection products, MC.

1. Introduction

The microorganisms are internationally recognized to play a pivotal role as ecosystem service suppliers [1-4]. Indeed, to accomplish their roles, microorganisms provide the turnover of organic matter in soil, mobilize plant nutrients and establish tight or loose relationships with plant roots (such as symbiotic nitrogen (N) fixation of legume crops with rhizobia, mycorrhizal symbioses, biocoenoses between cereal crops and *Azospirillum* spp.), thus contributing to plant growth by providing essential nutrients (e.g., N, C, P), water and phytostimulatory substances. In addition, microorganisms contribute to plant quality by altering (e.g., often enhancing) their nutritional and nutraceutical traits [5-7], and finally they can contribute to plant health by antagonizing harmful organisms [8]. Furthermore, microorganisms significantly reduce soil non-synthetic toxicants via their bioremediation potential [9, 10], contribute significantly to soil functional biodiversity, act as primary agents in the biogeochemical cycles, facilitate the carbon sinks build-up and contribute to animal welfare and nutrition [11]. There are several ways in which microbial activities can help plants to grow better and healthier, as it has been demonstrated in the last two decades by using conventional and molecular approaches. These include: (1) the production of secondary metabolites which are toxic to pathogens; (2) the induction of host plants to produce secondary metabolites which are toxic to pathogens; (3) direct hyper-parasitism of a single microbial strain towards one harmful organism; (4) the competition with plant

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pathogens for trophic/spatial niches; (5) the induction of resistance in the crops; (6) the alteration of the fertilization status and chemical traits of the host plants. This paper aimed at identifying the scientifically-based pros and cons of the European legislative framework about the borderline products based on microorganisms between bio-fertilizers/ bio-effectors and plant protectants. The paper focused in particular on the role of the microbial consortia (MC), which on the basis of a wider scientific evidence has gained in the last few years more popularity than the "microbials" based on single strains, and aimed at defining the boundaries of the two groups of microbial products, actually undefined, on scientifically-based criteria.

2. Microbes as Single Strains or MC

Very seldom microbes occur, survive and persist as single cells, strains or even single species in bulk soil, in the proximity of plant root canopy, as phyllospheric bacillus (i.e., on the plant leaf) or even as endophytes (i.e., present within the plant shoot or other parts) [12]. Most commonly, if not generally, they occur as members of a more complex microbiota [13], just as it happens in the human intestine [14], the stomach of ruminants [15], a biogas digestor [16], grape must and beer fermentation [17-19], cheese ripening [20], a legume root nodule [21], a termite nest [22-24] or in bio-mineralization mediated by anaerobic methane-consuming cell consortia [25]. Despite their intrinsic diversity, MC tends to respond to the environmental stressors as a unique organism, because they can have more chances than any microbial strain living as a single population to adapt one or more of their components to the stressor and can take advantage of internal beneficial interactions among members. Since each of a given ecosystem's physiological functions can be carried out by more than one microbial species, the functional biodiversity and the possibility of replacement among different microbial components play a fundamental role in maintaining an active life of the ecosystem. Furthermore, the balance among the different components of a MC, in quantitative terms, will ultimately consist of a continuous shift between actively growing (i.e., viable and culturable) cells and non-dividing (i.e., viable but non-culturable) cells of the various components of the total population. It is known that different microbial populations in a given environment "talk" to each other (e.g., through the "quorum sensing" mechanism) by exchanging precise chemical signals [26]. Natural MC holds many appealing properties also in a bioprocessing context, such as stability, functional robustness and the ability to perform complex tasks. The powerful features of natural consortia have inspired an interest in engineering synthetic consortia for industrial biotechnology applications [27].

3. Microbes Used as Bio-fertilizers/ Bio-effectors and Plant Protectants

For more than the last two decades (except rhizobia, a bio-fertilizer in use since 1896), dozens of plant protection products and a few bio-fertilizers have been used based on single microbial strains. More recently, the MC is receiving increasing attention for use in agriculture and agro-industry as either bio-fertilizers/bio-effectors or plant protectants. Single strains used as plant protectants are produced at high cell density in the formulated products and are subjected to extensive risk assessment [28]. The new regulatory framework for plant protection products is laid out in Commission Regulation (EC) No. 1107/2009 [29] and Commission Regulation (EU) No. 283/2013 [30], and explicitly requires consideration of impacts on non-target species, their ongoing behavior and the biodiversity and ecosystem, including potential indirect effects via alteration of the food web. Single microbial strains and thereof products used as bio-fertilizers or bio-effectors are present on the EU market as high cell density commodities and are subjected to risk assessment in the framework of

national legislations. These products generally do not require the same extensive risk assessment as for plant protectants and are therefore marketed with more limited registration requirements.

The density (expressed as CFU/g of product) of each of the components of a MC used as plant protectant remains considerably lower than the density which characterizes the products based on single strains, and several endpoints fall below the threshold of toxicological concern in the authorization process. However, at the moment, plant protectants consisting of MC should undergo the same procedures as the products based on single strains, despite the fact that it is scientifically very hard to imagine that the overall risk can be assessed basically as a summation of the risk of each of the components, i.e., without taking into consideration the interactions.

Bio-fertilizers/bio-effectors consisting of MC have the same registration requirements as the ones based on single microbial strains. The overall matter appears even more complex when considering that: (1) some microorganisms, either as single strains or as members of a MC, can have both effects, i.e., as bio-fertilizer/bio-effectors and plant protectants; (2) some microorganisms, having a potential for acting as plant protectants, play instead a role as members of a consortium in the organic matter turnover in soil environment or in a composting process. A few soil and rhizosphere microorganisms, such as *Bacillus subtilis*, are quoted in the annexes of the EU Regulation 1107/2009 [29], which deals with plant protection products. This has generated considerable uncertainty in the interpretation of the provision: if any *B. subtilis* strain must be considered functionally equivalent to a phyto-pharmaceutical, then any plant (with its rhizosphere where this bacterial species is quite common) or organic manures (where this species is also commonly present) should also be considered a plant protectant, thus requiring strict risk assessment and registration to be delivered or marketed. In addition, if a microbial species is quoted as such in the abovementioned regulation, all the strains of that species actually should not be marketed for purposes other than plant protectants, such as a bio-fertilizer or a component of a yogurt. This is in a sharp contrast with the requirements for registration of active microbes as plant protectant, which is always at strain level and not at species level.

At the moment, it appears that the intention of use and the mode of action should play a major role in the process of making decision about the categorization of the microorganism and the related registration requirements in the EU. In the authors' view, the third parameter affecting the assignment to the category of plant protection products or to the one of bio-fertilizers/bio-effectors is the cell density in the product to be marketed. The example is provided by composts, including mature manure compost, which are characterized by the presence of MC formed by strains with clear activity as bio-effectors (for both plants and functional diversity in soil) and strains with suppressive potential towards common plant diseases. As a logic consequence, the mature manure compost, which has the longest history of use as a bio-fertilizer in agriculture since thousands years, should be nowadays submitted to the registration procedures after undergoing the extensive risk assessment of the plant protection products, owing to its potential to help crops to grow better and healthier. This situation clearly represents an open question for industry and regulators, and offers a good opportunity for further improvement of scientifically-based registration procedures.

4. MC Is Different from Plant Extracts

As a consequence of the above considerations, it is advisable, for normative purposes, to clearly distinguish the bio-effectors in two sub-categories. One is represented by plant/algae extracts and one is represented by microbial single strains/consortia. The latter sub-category is internationally designated with the generic name of "bio-fertilizers", which very well

defines their nature and functions. Based on the different nature and origin, the two sub-categories clearly require different analytical and methodological approaches. Plant/algae extracts methods are widely available [31, 32], while for bio-fertilizers, some methodology is already included in national legislations, e.g., the Italian Fertilizers Act [33], in particular for products based on mycorrhizal consortia and other rhizospheric microorganisms. For the MC, high-throughput DNA sequencing has been proven invaluable for investigating diverse environmental and host-associated microbial communities. Recently, Franzosa et al. [34] have comparatively discussed the emerging strategies for microbial community analysis that complement and expand traditional metagenomic profiling. These include novel DNA sequencing strategies for identifying strain-level microbial variation and community spatial and temporal dynamics, for measuring multiple "omic" data types that better capture community functional activity, such as transcriptomics, proteomics and metabolomics, and for combining multiple forms of "omic" data in an integrated framework. The "multi-omics" approach has led to improved mechanistic models of microbial community structure and functions.

5. Case Studies of MC

5.1 Mycorrhizal Inoculants as Bio-fertilizers

Since mycorrhizal plants are more efficient in the uptake of specific nutrients in exchange of plant-assimilated carbon [35], arbuscular mycorrhizal fungi (AMF) inoculation of plants offers the possibility of reducing fertilizer applications. Therefore, AMF has gained popularity as "bio-fertilizers" both in the field [36, 37] and containers [38, 39]. A recent meta-analysis [40] of 38 published field trials with 333 observations to determine the effects of inoculation and root colonization by inoculated and non-inoculated (resident) AMF on P, N and Zn uptake. The growth and grain yield of wheat has shown that field AMF inoculation increases aboveground biomass, grain yield, harvest index, aboveground biomass P concentration and content, straw P content, aboveground biomass N concentration and content, grain N content and grain Zn concentration. Indeed, grain yield has been shown to be positively correlated with root AMF colonization rate, whereas straw biomass was negatively correlated. The most important drivers of wheat growth response to AMF have been shown to be soil inorganic matter, pH, total N and available P concentration, texture of soil, as well as climate and the AMF species inoculated. The meta-analysis shows that AMF inoculation of wheat in field conditions can be an effective agronomic practice, although its economic profitability should still be addressed for large-scale applications in sustainable cropping systems. The industry of mycorrhizal inoculants production is expanding around the world (e.g., http:// mycorrhiza.ag.utk.edu/). Although some studies indicate that inoculation with more than one AMF isolate may not bring more benefit to the host plant [41], a mixture of AMF with complementary functions appears to be more beneficial to the plant than a single isolate [5, 42]. In the field [43], performances of *Trifolium alexandrinum* inoculated with the exotic AMF, both single and mixed, were compared to those obtained with a native inoculum, showing that field AMF inoculation increased crop productivity and quality and that the native inoculum was as effective as, or more effective than the exotic AMF isolates. The persistency of the beneficial effects of AMF was also shown until the second year after inoculation with yield increases of the following crop (maize). In a second field study, Pellegrino and Bedini [6] tested the agronomic relevance of field-inoculated locally sourced and foreign inocula on chickpea (*Cicer arietinum* L.), one of the most important worldwide grain legumes, evaluated not only the yield but also the improvement of the nutritional value of chickpea grain by protein, Fe and Zn bio-fortification and

showed in the field the role of AMF as bio-fertilizers of crops and bio-fortification tools. Recent advances have shown that MC containing mycorrhizal inocula is more effective [44, 45]. The patented commercial product "Micosat F" (MF) contains a mixture of AMF (*Glomus coronatum*, *G. caledonium*, *G. intraradices*, *G. mosseae*, *G. viscosum*) and helper bacteria (*Pseudomonas* spp., *Bacillus* spp., Actinobacteria *Streptomyces* spp. and the saprophytic fungi *Trichoderma* spp.). Delivery of the inoculant is done via roots or seed coating, and for trees through localized soil treatment with granular formulations. The use of the AMF alone and the MC of MF was comparatively studied on some major crops to measure the quantitative response and final quality of the epigeal parts. The quantitative response on average was higher for MF: for maize 19% in cut up, 12% in spikes with bracts and 6.4% of grain yield; 13% for wheat grain; 11% for total yield of tomato, due to an increase of 6% of the fruit mass; 11% for cucumber; 8%-20% in the development of the olive trees; null in melon a normal mycotrophic species [45]. The rapid scan by UV-Vis-NIR rays from 350 nm to 2,500 nm of the leaves, flower and fruit parts, which was associated with a rapid examination by an electronic nose (EN) for a total of more than 1,400 analyses, revealed that the cultures submitted to the microbial treatments appeared different from the control samples, with linear regression *R* values of 0.40-0.70, but with oscillations between the different species and run-test. Grain- and forage-maize, aromatic plants, camellia, apple (flowers and leaves), melon and water melon, ryegrass *Lolium* spp., oat and clover are strongly responsive to the treatment with the MC. Tomato was mediumly respondent, while alfalfa and vetch were lowly respondent in an EN test. In some cases, the results of the rapid methods were fairly corroborated by fine chemical analyses. The modern wheat cultivar "Blasco" treated with MF gave consistent results, predicted by the EN test, in a bread-making panel test: the panel appreciated the bread from the treated "Blasco" flour as very similar and as good as the bread obtained from the ancient wheat cultivar "Sieve", "Inallettabile" and "Gentil Rosso" [45]. Another study on tri-trophic consortium *Azospirillum-Pseudomonas-Glomus* [46] showed that the three-component inoculants may be useful in promoting maize growth. Application of a consortium of AMF and the plant growth-promoting rhizobacteria (PGPR) was studied by Mäder et al. [47] and found to positively affect crop yield, grain, soil quality and nutrient uptake of the staple food crop wheat (*Triticum aestivum* (L.)) in a rotation with either rice (*Oriza sativa* (L.)) or black gram (*Vigna mungo* (L.) Hepper). Recently, Berta et al. [48] have shown that the inoculation with MC containing bacteria and AMF promote the growth of maize cultivated in field conditions and differentially affect the grain nutritional content.

The induction of a healthier status of crop plants (e.g., increased content protein, starch and microelements) due to the usage of MC containing AMF may encompass a natural, decreased susceptibility of the plants to pathogens. It has been proposed to call this trait "mycorrhiza-induced resistance (MIR)" [49-51], providing systemic protection against a wide range of attackers and sharing characteristics with systemic acquired resistance after pathogen infection and systemic induced resistance following root colonization by non-pathogenic rhizobacteria. It is commonly assumed that fungal stimulation of the plant immune system is solely responsible for MIR. However, the latter could be the result of a cumulative effect of direct plant responses to mycorrhizal infection and indirect immune responses (ISR) to ISR-eliciting rhizobacteria in the mycorrhizosphere. The mycorrhizal MF-induced resistance has been verified in the case of flavescence of vines in the Pedimont area in Italy [52]. Continuous cropping of vines in the same soils during the last 70 years and over-usage of chemical fertilizers has produced the well-known soil degradation effects on

one side [53] and a generalized impairment of vines towards phytoplasms responsible for the so-called "flavescence". In two different farms (Santa Caterina in Grazzano Badoglio and Torelli in San Grato di Bubbio, both in the Province of Asti), which had 30% of the vines affected by flavescence, the new vines in the first farm were soil-inoculated with the MC, and the old vines were equally treated in order to re-establish the microbial functional biodiversity in soil [52]. In both cases, the entire area has become flavescence-free and still is after 10 years from treatment, despite the presence of the latter in all the surrounding vine-farms.

5.2 Composts as Bio-fertilizers

During composting, microbial decomposition aerobically transforms organic substrates into a stable, humus-like material [54]. The composted organic matter is an excellent tool to contrast soil erosion and desertification, and there is a generalized need to provide these soils with an adequate return of the organic carbon subtracted by the continuous cropping. When deeply humified, compost can substantially help in contrasting the increased carbon dioxide emissions. In EU Southern zone, where the majority of soils contain less than 2% organic matter, and more in general in the entire Mediterranean Basin, the wet olive husks provide annually 20-30 million tons of biomass, which could help contrasting the continuous deprivation of organic matter due to intensive agricultural management. This end use of wet olive husks can be achieved through composting, which is a less environmentally impacting process compared to the production of electric power or heat as end uses [55]. Recently, Echeverria et al. [56] have described a method to industrially transform the wet olive husks as a sole raw material in a high quality "green" compost by using MC as starters. The compost produced in 60 d is a mature, deeply humified organic matter useful to restore soil fertility and soil texture in both agriculture-intensive and less intensive areas. In addition, it has been found to effectively substitute for turf as a cultivation substrate in horticulture at greenhouse level, with beneficial effects on nutraceutical traits of tomato fruits. The composting process can be run by using the same MC as starters, also at farm scale [57], and the final product is equally characterized by a high microbial biodiversity. The use of "green" composted amendments should be encouraged, also considering the continuous decrease of organic matter content in agricultural soils. Incidentally, apple orchards treated with this soil amendment, in which the MC contains bacteria, such as *Bacillus amyloliquefaciens* subsp. *plantarum* and microfungi like *Trichoderma atroviride*, proved to be healthier, e.g., not affected by the attacks of *Alternaria alternata* than all surrounding orchards which were severely affected by the fruit- and leaf-spots. Similar observations were made by Alfano et al. [58] for another compost obtained from olive mill waste. This type of indirect disease control, which includes the activation of induced systemic resistance in the plants by the microbial compost population and the improved plant nutrition and vigor leading to enhanced disease resistance, is clearly different from a direct plant disease control, and looks like a secondary non-tailored effect compared with the primary effect of the amendment as a bio-fertilizer.

5.3 Suppressive Composts as Plant Protectants

Direct biological control of soil-borne plant pathogens by suppressive composts now is an established horticultural approach [59]. The mechanisms of direct disease control suggested for disease suppression by composts, include direct parasitism of pathogens and competition for nutrients, such as carbon or iron and antibiosis. The delivery mode of the biological control agent is its addition and subsequent sorption of the plant protectant to a compost. Plant growth media enriched with the biological control agent *Trichoderma asperellum*

strain T-34 reduced the incidence of *Rhizoctonia solani* disease. In composts aged 0.5-1 year, this strain was only efficient when added to spent mushroom and cork compost, although it remained well established in all of them. The fact that strain T-34 transformed all composts aged 1.5-3 years into highly suppressive composts was attributed to the low levels of easily biodegradable substances. *Rhizoctonia* damping-off in cucumber plants can be reduced by using composts and/or the biological control agent *T. asperellum* strain T-34. In addition, the extent, to which the composts suppress this disease, depends on the chemical-physical nature of the composted materials and increases with the compost maturity [60]. More in general, composts can be transformed into suppressive compost by the addition of one or more biological control agent, specifically active against a plant disease. Fortifying composts with beneficial microorganisms is one possible factor that can help increasing the efficacy and reliability of disease control [61]. The distinction between bio-fertilizers/ bio-effectors and plant protectants is clearly reported by SANCO [62]. However, a few soil and rhizospheric microbial species are mentioned in the EU legislation [29] as bio-pesticides, along with the many chemicals for use as phyto-pharmaceuticals, collectively called “plant protection products”. The uncertainties deriving from this quotation of given microbial species (then all strains of this species should be considered plant protection products even if they have nothing to do with protection of plants) should be solved as soon as possible. Indeed, an incorrect interpretation of this provision can negatively affect common agricultural practices at EU level (e.g., usage of manure and composts as bio-fertilizers, use of MC as bio-fertilizers) and the human welfare via the nutritional value of food, considering that human microbiota depends also on the quality of the MC of different foods [63]. A depression of microbial diversity of our intestine may cause important human pathologies, such as diabetes type II or inflammatory processes. A well balanced nutrition and the correct presence of microbiota in the intestinal tract of humans and animals increase their natural defense potential [64] without being considered *per se* a medicine. The same logics should be used for plant health, allowing the use of beneficial MC not having a specific activity against plant pathogens, such as bio-fertilizers, simply because they promote plant growth and plant nutrition. Therefore, in agreement with Malusà and Vassilev [65], it seems relevant at normative level: (a) avoid the interpretation of EU Regulation 1107/2009 [29] that any strain belonging to a quoted species must be considered a plant protection product, therefore not allowing the usage different strains belonging to the same species for other purposes, such as bio-fertilizers; (b) promote a sensible legislation at EU level of bio-fertilizers (i.e. fertilizers based on living microorganisms), which in turn would stimulate their industrial production, thus setting appropriate qualitative standards and defining a science-based risk assessment; (c) consider that bio-fertilizers can promote the intrinsic ability of plants to counteract abiotic stresses, e.g., drought [66] as well as biotic stresses, e.g., plant pests [67, 68] without being *per se* phyto-pharmaceuticals, but rather beneficial rhizospheric microorganisms; the microbial strains or consortia would not necessarily need to be registered as plant protectants, but could be registered as bio-fertilizers or bio-effectors, with an appropriate risk assessment.

6. MC as Bio-effectors: An Approach to Risk Assessment

In order to provide a frame for an approach to risk assessment of MC to be marketed in EU as bio-effectors, the following elements should be taken into consideration: (1) the MC should be evaluated and properly assessed before entering the market; (2) the risk assessment should take into consideration on the features and traits of MC, which are different from the ones characterizing microbial products based on

single strains at high density. To achieve this goal, the product based on MC should be assessed by: (1) describing in full the taxonomically identified components of the MC and their biological traits; (2) describing the physical-chemical traits of the product and the methods used, including statistical evaluations; (3) assessing the toxicological traits as a whole consortium with respect to the production of (secondary) metabolites of toxicological concern *ex planta* as well as *in planta* after short/extended exposure of the plant to the MC at mesocosm (greenhouse) level, i.e., looking at whether a (rhizospheric) soil extract contains relevant metabolites in concentration of toxicological concern; (4) describing the efficacy of the consortium; (5) describing the invasiveness and persistence of the MC in the environmental compartments for an appropriate number of crop cycles; (6) identifying the ecotoxicological consequences of the use of the MC on ecologically relevant non-target species or non-target species providing ecosystem services.

7. Conclusive Remarks

In the delicate normative balance of the borderline products (plant protectants and bio-fertilizers/ bio-effectors), the intention of use was mentioned in the first instance and the mode of action was in a second place. For the latter, the basic difference between the two type of products is that a plant protectant mostly has a targeted activity on plant pathogens, while a bio-fertilizer acts indirectly by fortifying the host plant to make it become a healthier plant, thus inducing a generalized resistance to the onset of pathological status, irrespective of its origin and nature. Therefore, bio-fertilizers exhibit a double effect—biotic and abiotic, leading to the fortification of the crop plant linked to its more effective water and nutrients uptake, as well as to a generalized healthier status. This in turn means a higher resistance to diseases. In addition, bio-fertilizers play a relevant role for the reduction of the environmental impacts of chemical fertilizers by facilitating the uptake of P, thus reducing the need of P fertilization. Actually, the reservoirs on the earth are expected to extinguish by the year 2050, at the actual rate of extraction/ consumption. Although finding a scientifically-based balance between regulatory needs and marketing constraints is not always an easy task, the availability of scientific advancements combined to common sense should help in describing the risk profile of MC satisfactorily for all interested parties.

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Improvement of Physical and Biological Quality of Soil in a Sugarcane Plantation through the Management of Organic Matter Input

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Abstract: Changes in soil quality of sugarcane plantation as a result of changes in land management can not be measured directly, but must be demonstrated by measuring the change in the properties of the ecosystem as an indicator. This research aimed to study the effect of the addition of various quality and quantity of organic matter on soil biology (earthworms) and physical quality (aggregate stability, macroporosity and infiltration rate). There were 15 treatment combinations tested. The first factor is the type of organic matter: (1) cattle manure (CM), (2) filter cake (FC), (3) sugarcane trash (ST), (4) a mixture of CM + FC and (5) a mixture of CM + ST. The second factor is the application dose of organic matter, which consists of three levels—5, 10 and 15 Mg/ha. The treatments were arranged in a factorial randomized block design with three replicates and one control treatment (without organic matter input). The result of this research showed that the highest population density of earthworms was found in the treatment of ST (78 individuals/m^2) and a mixture of CM + ST (84 individuals/m^2). The type of organic matter with C/N ratio ranged from 15.5 to 34.7 and cellulose content in 33.3%-40.1% gave better growth of earthworm. The effect of increase in earthworm growth on soil physical improvement is more apparent in the treatment of mixture of low quality and high quality organic matter. The increase of earthworm density and biomass enhanced soil macroporosity (from $r = 0.683$ to $r = 0.606$) and infiltration rate (from $r = 0.669$ to $r = 0.756$). The results of this study suggest a mixture of CM + ST or ST alone as organic matters, which is recommended to improve soil physical and biological quality of sugarcane land, with the dose application ranged from 10 Mg/ha to 15 Mg/ha.

Key words: Quality and quantity of organic matter, earthworms, physical and biological quality of soil.

1. Introduction

Managing soil organic matter content is very important for maintaining nutrient cycling in agroecosystem, improving soil physical condition and maintaining a healthy environment [1]. Organic matter plays an important role in regulating of nutrient flux and microbial biomass [2] and improving soil physical properties [3, 4], chemical properties [5] and biological properties [6]. Increase of soil C-organic content can directly improve soil structure as shown by water-stable aggregates [3, 4]. However, improvement of soil structure can also be caused by the indirect effect of organic matter through increasing population density of soil dweller type of earthworm and plant roots density resulting a better soil structure, soil porosity and water infiltration [7, 8]. Therefore, low input management in agroecosystems got a lot of attention from sugarcane farmers in Indonesia, especially in East Java. Hairiah et al. [9] reported their research result in an ultisol in North Lampung that the application of 16 Mg/ha bagasse (sugarcane processing waste) and 8 Mg/ha sugarcane trash (harvest residue) increased sugarcane production of 18-21 Mg/ha with the average sugar content of 7%-8% (relative to total dry weight), and the sugar production was about 1.5 Mg/ha. Some findings also had been reported that the applications of organic

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matter, such as cattle manure, crop residues and compost, can improve the status of soil organic matter, soil structure and soil fertility [10, 11]. The soil quality of the agricultural land that received anorganic input is better than conventional farming systems [12]. However, the effects of organic matter on soil quality are differed depending on its quality. According to Palm and Sanchez [13], organic materials with low C/N ratio (< 25), low concentrations of lignin (< 15%) and polyphenolics (< 3%) are considered to be high-quality, meaning material decomposes and nutrients are released rapidly. Research about the effect of different quality organic material on soil quality improvement in sugarcane plantation, however, is rather limited.

This research aimed to study the effect of different quality of organic material on the soil quality as measured by changes in the biological (earthworms) and physical soil quality (aggregate stability, soil macroporosity and soil infiltration rate). Hypotheses of this study are: (1) the application of a mixture of high and low quality organic matter will give better earthworm growth than a single quality of organic matter, (2) the increase of the application rate of organic matter will increase the growth of earthworms and (3) the increase of earthworms growth gives the improvement of soil physical properties.

2. Materials and Methods

2.1 The Experimental Site, Climate and Soil Characteristics

The field experiment was conducted on an inceptisol soil type for one year (during planting season) at Sempol village, Pagak sub-district, Malang regency (08°16.837′ S and 112°30.453′ E, 424 m above sea level). It was initiated in November 2010 to December 2011 in rainy season until dry season of 2010-2011. The climate of the experimental site is tropical with rainy season (November-May) and dry season (June-October). The average annual rainfall was 1,199 mm, while the average annual temperature was 25.3 °C. The soil of the experimental site is loam and has the following properties: 26% of clay, 48% of silt and 26% of sand. It is well drained and flat, and has bulk density of 1.24 Mg/m^3. The soil is very low in organic carbon (1.06%), with pH (H_2O) = 5.2 and pH (KCl) = 4.5, low in total N (0.16%), low in available P (9.17 mg/kg), medium in exchangeable K (0.54 meq/100 g) and medium in cation exchange capacity (CEC, 23.23 meq/100 g).

2.2 Treatments

The treatments were arranged in factorial block randomized design. The first factor is organic matter source that consists of five different quality of organic matter, i.e., cattle manure (CM), filter cake of sugar mill (FC), sugarcane trash (ST), mixture of cattle manure + filter cake (CM + FC), and mixture of cattle manure + sugarcane trash (CM + ST). The second factor is three application rates of the organic matter which are 5, 10 and 15 Mg/ha. The combination of two factors made 15 combinations of treatments plus one treatment (no organic input) as a control. Each treatment was replicated three times.

2.3 Preparation of Organic Matter and Analysis of the Organic Matter Quality

The used organic matter was two weeks been composted. Sugarcane trash was collected after harvesting and then was ground (< 2 mm). All organic matter samples were analyzed in laboratory for total N (by Kjeldahl digestion), C-organic content (by Walkley Black), lignin, cellulose and ash content by Goering and Van Soest [14], polyphenols content (by Folin-Denis) and gross energy (by Bomb calorimeter method). The results of these analysis were presented in Table 1.

2.4 Earthworms Inoculation

Earthworm *Pontoscolex corethrurus* obtained from coffee plantation, was inoculated into the planting hole in one week after organic matter application.

Table 1 The chemical composition of organic matter on dry weight basis.

Organic matter	Total C-organic (%)	Total N (%)	C/N	Lignin (%)	Ash (%)	Cellulose (%)	Polyphenol (%)	Gross energy (kcal/kg)
CM	16.7	1.94	8.30	12.3	13.3	30.3	0.26	1,011
FC	20.2	1.98	10.2	19.9	20.5	40.2	1.14	1,090
ST	28.1	0.81	34.7	13.3	10.2	40.1	2.01	3,028
CM + FC	19.2	1.68	11.4	16.5	11.5	37.5	1.42	1,120
CM + ST	20.4	1.32	15.5	12.0	8.22	33.3	1.12	1,354

Before the inoculation, a plastic barrier was installed among experimental plots to avoid any movement of inoculated earthworm. Each plot was inoculated by 125 individuals of earthworm with average weight per individual ranged from 0.2 g to 0.4 g. After the inoculation of earthworm, the soil surface was covered by sugarcane trash to avoid direct sunlight.

2.5 Crop Culture

The plots size of 10 m × 1 m was prepared by hoeing for all treatments uniformly. The sugarcane cultivar Bululawang-red with one bud and 10 cm length was planted in seedling beds for a month to obtain uniform seedlings. Subsequently, they were transplanted into the soil beds with planting distance of 40 cm inter-plants. During growing season, there were no chemicals (herbicide, pesticide or insecticide) applied. All the organic amendments were manually applied to field plots one month before planting. In addition to organic matters used for the treatments, the soil also received basic fertilizers NPK (15:15:15) of 200 kg/ha and ammonium sulfate of 800 kg/ha. The fertilizers were applied one month after transplantation by band application on distance of 10 cm from the plant.

2.6 Earthworm Sampling and Measurement

The population density of earthworms was determined by taking 48 soil monoliths (25 × 25 × 20 cm size) samples, each point was chosen in between two sugarcane plant of each plot, at soil depths of 0-10, 10-20 and 20-30 cm, according to a sampling procedure described by Huising et al. [15]. The earthworm samples were collected by hand sorting and calculated on population density (*D*, individuals/m^2), and weighed for its fresh weight (biomass, g/m^2). Weight per individual was estimated by the earthworm's biomass and density ratio (*B/D*). The earthworm measurement was conducted in April, July and December (during rainy and dry season).

2.7 Soil Sampling and Soil Physical Analysis

Soil samplings were taken in each treatment of each plot at one, three, six and nine months after planting. The measurements of stable aggregate were done using aggregate soil sample with diameter of > 5 mm, while for soil macroporosity measurement, an undisturbed soil sample was taken using a ring sample (4.5 cm diameter, 5 cm height). Aggregate stability was determined by wet sieving method and calculated it mean weight diameter of the aggregate (mm). The macroporosity was obtained by calculating soil water content in $pF = 0$ minus $pF = 2.5$ [16]. Infiltration rates were measured using a falling head single ring infiltration with diameter of 20 cm that was inserted to a depth of approximately 10 cm. The infiltration tests were conducted for at least 3 h until the infiltration rates were found to become constant. Cumulative infiltration was plotted against time and the data were fitted the Philip's infiltration equation [17].

2.8 Statistical Analysis

The collected data were statistically analyzed by using analysis of variance (ANOVA) (*F*-test) at level $P \leq 0.05$ and differences in each treatment were adjudged by least significant difference (LSD) test ($P \leq 0.05$) using program Minitab version 14.12. For statistical analysis of data (charts), Microsoft Excel

was employed.

3. Results and Discussion

3.1 Effect of Quality of Organic Matter on Earthworm

The result of this study showed that the addition of different quality of organic matter has significantly ($P < 0.05$) affected the earthworm population densities (Table 2). The average increase of earthworm population density compared to control (no organic input) were 90% for CM, 106% for FC, 118% for ST, 65% for CM + FC and 135% for CM + ST. Mean weight per individual of earthworm (g/individual) calculated from the ratio earthworm biomass to population density tends to increase with increasing the applicated doses of organic matter. The application of the mixture of CM + ST and ST alone at a dose of 5-15 Mg/ha gave the highest population density (Fig. 1).

The increase of earthworm biomass on the plots which received the addition of organic matter to control were 165% for CM, 235% for FC, 273% for ST, 212% for CM + FC, 358% for CM + ST (Table 2). The increase of organic matter application rates increases earthworm biomass, except in the plot which received the mixture of CM + FC (Fig. 1). This result indicates that the organic matter input was needed to increase earthworm biomass, and differences of organic matter quality will affect the increase of earthworm biomass, when environmental conditions are less suitable for the growth of earthworms. It caused the different increase patterns of earthworm biomass with increase of the application rates. The addition of organic residues into the soil is a source of food and energy for soil biota [18, 19]. The result of this research is in line with which reported by previous researchers confirmed that the residue management either the mulch or the cover crop increased

Table 2 Effect of addition of various organic matter on earthworm population density (*D*), earthworm biomass (*B*), mean weight diameter of aggregate (*MWD*), macroporosity and infiltration rate.

Treatments	*D* (individuals/m^2)	*B* (g/m^2)	*B/D* (g/individual)	*MWD* (mm)	Macroporosity (%)	Infiltration rate (cm/h)
Control	35.6^{a}	2.60^{a}	0.073^{a}	0.65^{a}	8.65^{a}	31.2^{a}
CM	67.6^{bc}	6.90^{b}	0.102^{ab}	0.99^{b}	9.87^{b}	35.4^{b}
FC	73.5^{cd}	8.70^{bc}	0.118^{bc}	1.20^{c}	9.84^{b}	39.6^{c}
ST	77.6^{cd}	9.50^{c}	0.122^{bc}	1.31d	10.50^{c}	43.7^{e}
CM + FC	58.7^{b}	8.10^{bc}	0.138^{c}	1.28^{cd}	10.20^{bc}	42.2^{d}
CM + ST	83.6^{d}	11.90^{d}	0.142^{c}	0.99^{b}	10.30^{c}	43.6^{e}
LSD ($P = 0.05$)	10.7	1.90	0.030	0.08	0.44	1.01

LSD = least significant difference; $^{a-e}$means followed by the same letters at each column are not significantly different ($P = 0.05$).

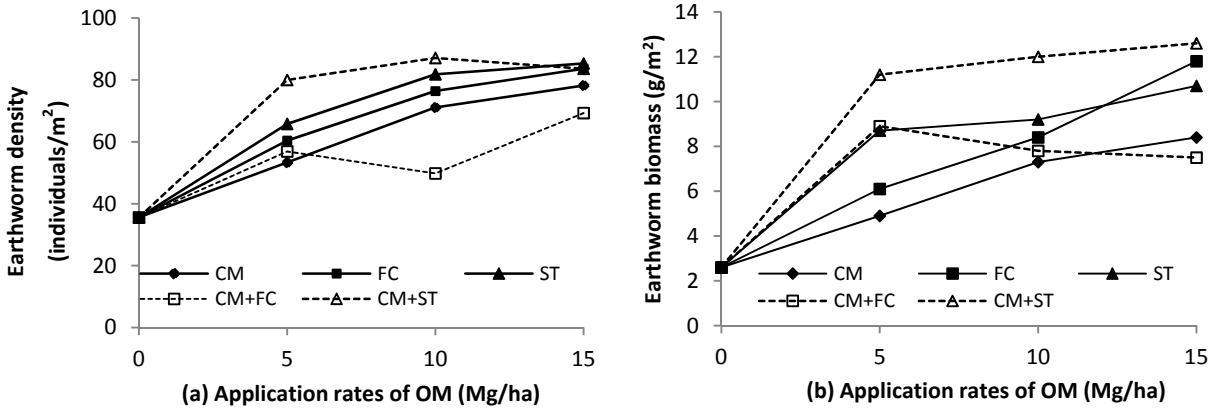

Fig. 1 Effect of application dose of various organic matter (OM) on the average earthworm density (a, LSD = 18.40) and biomass (b, LSD = 3.42).

population density earthworms ranging from 18.5 individuals/m^2 to 451.2 individuals/m^2, and earthworm biomass ranged from 1.3 g/m^2 to 142.3 g/m^2. The residue left on the soil surface can increase the biomass of earthworms 2.9 fold in fallow soil [20-22].

The palatability of earthworm food determines the growth and development of earthworms. Schönholzer et al. [23] reported that the organic matter quality that measured by C/N ratio greatly determined palatability of organic matter consumed by earthworms. The residue consumption level of earthworm was positively correlated with the C/N ratio on the range of 12-39. Residues with C/N ratio of 12.3 are preferred than C/N ratio of 8. Neilson and Boag [24] and Valckx et al. [25] reported that the grasses residue with the same palatability on the C/N ratio of 11.4 to 15 is more consumed by earthworms. Increase in the application rates of organic matter increases the population density and biomass of earthworms. García and Fragoso [18] reported that the growth and biomass of earthworms, including *P. corethrurus*, was influenced by the quality and quantity of food available in the soil. The higher the quantity of applied organic matter is, the larger the amount of energy and food resources is available to earthworms, thereby increasing earthworm populations and activity.

3.2 Effect of the Organic Matter Quality on Soil Physical Properties

The quality of organic matter influenced significantly ($P < 0.05$) *MWD* of soil aggregate. The average increase of soil aggregate *MWD* of each the organic matter quality in comparsion with the control were 52% for CM, 85% for FC, 101% for ST, 97% for CM + FC and 52% for CM + ST. Increase of organic matter application rates can increase average *MWD* of soil aggregate, except on application of mixture of CM + FC (Fig. 2). This result suggests that the organic matter input even though low quality can enhance the formation of stable aggregates. It probably caused by that organic input enhanced soil biota activity in the organic matter decomposition process as well as earthworms. The result of organic matter decomposition process and biota activity can play an important role as a granulator in stable aggregate formation. Earthworms prefer the low quality of organic matter and high C/N ratio and cellulose content [22]. The results of decomposition

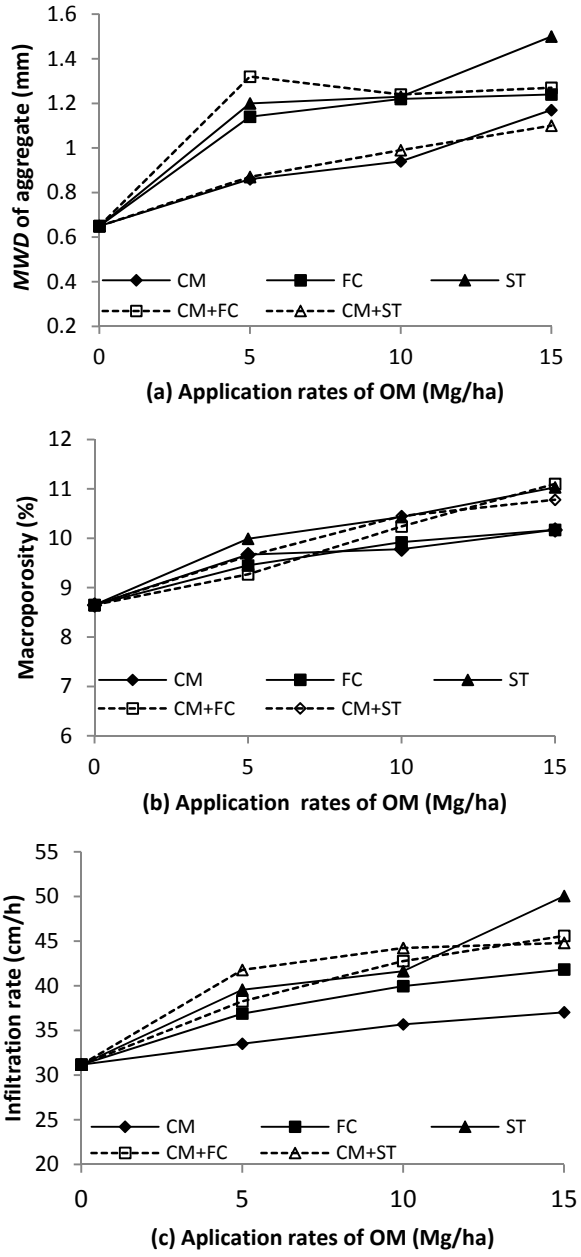

Fig. 2 Effect of application rates of various organic matter (OM) on *MWD* of aggregate (a, LSD = 0.14), macroporosity (b, LSD = 0.76) and infiltration rate (c, LSD = 1.76).

and cast formation can increase soil aggregate stability.

The results of this research also showed that the addition of various organic matter with different rates gave higher macroporosity than the control. The increase of organic matter application rates increases macroporosity with similar increasing pattern for each treatment (Fig. 2). The average macroporosity increase of the addition of various organic matter were 14% for CM, 14% for FC, 27% for ST, 18% for CM + FC and 19% for CM + ST, respectively (Table 2). These results suggest that the application of low quality of organic matter can increase the activity of soil biota to produce greater amounts macropore. It is caused by the low quality of organic matter, which has a high gross energy (Table 1). Availability of more food and energy resources can increase earthworm activity in burrowing and formation of holes and channels in the soil.

The difference of organic matter quality influenced significantly ($P < 0.05$) the soil infiltration rate. The increase of infiltration rate of each treatment compared to the control were 14% for CM, 27% for FC, 40% for ST, 35% for CM + FC and 40% for CM ST, respectively (Table 2). This result indicates that the application of low quality of organic matter with larger particle size due to more porous soil and higher soil infiltration rate. The higher organic matter application rate is, the higher soil porosity is, especially in the upper layers of soil. It caused increase of soil infiltration rate (Fig. 2). These results also suggest that input of low quality of organic matter can increase the infiltration rate due to increasing earthworms activity in formation of soil macropore.

Organic matter is the main agent of aggregate stabilization in some form, such as (1) the decomposition products of plant, animal and microbial residues, (2) itself microorganism and (3) the products of microbial synthesis, such as polysaccharide and gums that are formed during the decomposition of organic residues [26, 27]. The quality and quantity of residue affect the aggregate formation and stabilization [28].

The results of this study showed that the application of sugarcane trash (low quality of organic matter) at a rate of 15 Mg/ha gave a high *MWD* of soil aggregate. The increase in aggregate stabilization can occur because of stimulation of micro- and macro-fauna activity and soil microflora [27]. This study is in line with the one reported by Coq et al. [29] that the effect of earthworm activity on the formation of stable aggregates is larger in the treatment with the addition of low quality of organic matter than high quality of organic matter, such as legume crops residue. The increase in growth and population density of earthworm can stimulate the activity of other soil microorganisms [30], so it assists in the stabilization of aggregates. Earthworms' activity through their burrowing and casting activities also affected the soil porosity. Also known as "ecosystem engineers" [31], earthworms produce structural features at three different scales of soil porosity. They live and are active in the soil, and consume litter available in the soil or on the soil surface [8, 32, 33]. Earthworm activity can alter pore spaces between mineral and organic particles to influence the soil macroporosity and soil structure stability [34].

Lamande et al. [7] reported that the difference of land management will produce a different earthworm community. It will further influence the structure of the soil pore morphology to influence the water movement in the soil, as measured from hydraulic conductivity and soil infiltration rate. A good soil structure will help the movement and retention of water in the soil and improve crop rooting environment. Thus, maintaining organic matter inputs is needed to increase earthworm activity and improve the development of plant roots due to maintenance of soil porosity [8].

3.3 The Relationship between the Variables of Soil Biological and Physical Properties

The results of correlation analysis showed that the

Table 3 Correlation coefficient between earthworm variables and soil physical properties.

Variables	Earthworm population density (individuals/m^2)		Earthworm biomass (g/m^2)	
	r	*P*-value	*r*	*P*-value
MWD aggregate (mm)	0.416	0.110	0.416	0.110
Macroporosity (%)	0.683	0.004*	0.606	0.010*
Infiltration rate (cm/h)	0.669	0.005*	0.756	0.001*

*Means statistically significant difference at $P < 0.05$.

earthworms variables were positively correlated with the variables of soil physical properties (Table 3). This suggests that the greater the population density and biomass of earthworms are, the higher the average aggregates *MWD*, macroporosity and soil infiltration rate are. These results indicate that the addition of organic matter into the soil provides a direct influence on the population and activity of earthworms. Increased population and earthworm activity improved soil physical properties. The significant influence of the increase in population and activity of earthworm was shown in soil macroporosity and infiltration rate. This suggests the role of *P. corethrurus* endogeic earthworms group through their burrowing and casting activity in the subsurface layer is clearly visible.

The earthworms can improve soil aggregation through amendmenting biological and physico-chemical soil [34], as well as the direct effect and indirect effect on soil structure and content of soil organic matter [34, 35]. However, impact of earthworms on the aggregation varied depending on the quality of the organic matter residue added to the soil [36], because the population and diversity of earthworms were affected by the quality and quantity of organic residues [37]. The *P. corethrurus* earthworm (geophagus earthworm) can digest soil. It can destroy soil aggregates to make them become unstable. However, the biochemical process of soil digesting activity can stabilize soil aggregates [38]. Rearrangement of soil particles affected the water movement into soil (infiltration) [35]. Thus, earthworm activity increases soil aggregate stability, soil macroporosity and soil infiltration rate [36].

4. Conclusions

The addition of various quality of organic matter increased earthworm population density, respectively, 90% for CM, 106% for FC, 118% for ST, 65% for CM + FC and 135% for CM + ST, and earthworm biomass, respectively, 165% for CM, 235% for FC, 273% for ST, 212% for CM + FC and 358% for CM + ST compared with the control. It also increased soil macroporosity, respectively, 14% for CM, 14% for FC, 27% for ST, 18% for CM + FC and 19% for CM + ST, and the rate of infiltration by 14% for CM, 27% for FC, 40% for ST, 35% CM + FC and 40% for CM + ST than in the control. The addition of sugarcane trash with a ratio of C/N = 15.5, alone or mixed with high quality organic matter (CM + ST) with a ratio of C/N = 34.7 is likely to provide the improvement of biological and physical soil quality higher than the other treatments. The recommended application rate based on the results of this study was 10-15 Mg/ha. Increasing population density and biomass of earthworms improves soil macroporosity and infiltration rate. Thus, the addition of organic matter derived from sugarcane harvest residue alone or mixed, is recommended in the sugarcane land management for maintaining the existence and activity of earthworms in the sugarcane land. It is an important factor in the improvement of soil physical properties of sugarcane land.

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Analysis of Irrigation Systems Employing Comparative Performance Indicators: A Benchmark Study for National Irrigation and Communal Irrigation Systems in Cagayan River Basin

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Abstract: Comparative performance analysis of four irrigation schemes within Cagayan River Basin was assessed using comparative performance indicators between the years 2008 and 2012. The objectives were to establish benchmarks for both productivity and performance of irrigation schemes along the valley and to inquire whether small schemes function better than large schemes. The performance evaluation study of the systems composed of three general performance indicators, based on three domains—(1) system operation performance; (2) agricultural productivity and economics; (3) financial performance. Each indicator was assessed based on the prescribed descriptors used by the International Water Management Institute (IWMI) and Food and Agriculture Organization (FAO). Analysis showed an overall system performance efficiency of 59%, 55%, 47% and 36% for Magat River Integrated Irrigation System (MARIIS), Lucban, Garab and Divisoria Communal Irrigation Systems (CIS), respectively. In terms of annual productivity performance, Lucban CIS dominates the three other systems with 0.35 kg/m^3, which was classified as moderately performing system, while the rest were classified with low productivity index. Financial sustainability of the systems were extremely poor with cost recovery ratio of 0, 0.33, 0.41 and 0.49 for Divisoria, Garab, Lucban and MARIIS, respectively, which were exceptionally below the standard value of at least one. Also, analysis of the indicators revealed that on average, large schemes performed similarly to small-scale schemes, but small schemes were more variable, particularly in input-use efficiency. The benchmarking study will provide strategic information to policy makers of agricultural and irrigation agencies on the existing weaknesses of irrigation systems in the country and determine in a more quantifiable terms levels of potential improvement and intervention targets.

Key words: Communal and national irrigation systems, performance benchmarking, small and large reservoir schemes.

1. Introduction

Benchmarking is already widely accepted and advocated by several organizations worldwide, such as, International Program for Technology and Research in Irrigation and Drainage (IPTRID), International Water Management Institute (IWMI), International Commission on Irrigation and Drainage (ICID), World Bank (WB) and Food and Agriculture Organization of the United Nations (FAO), because it is a very powerful management tool for analyzing and improving the performance of water resources projects [1]. In the case of Magat River Integrated Irrigation System (MARIIS) and Communal Irrigation Systems (CIS), there has been an urgent issue to be addressed to improve the performance of these systems. Initial benchmarking on the current situation of the system using standard domains and indicators were considered to produce strategic information for effective short and long term measures and plan to manage water resources more efficiently, sustainably and productively, which enables managers to compare the processes with the best practices and adopt suitable ones which would eventually improve the efficiency of the system and will result in savings in

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water usage thereby increasing the systems coverage [2].

For this study, two irrigation domains were covered as follows:

(a) Service delivery: this domain includes two areas of service provision: (1) the adequacy with which the organization manages the operation of the irrigation delivery system to satisfy the water required and (2) the efficiency with which the organization uses resources to provide this service (financial performance);

(b) Productive efficiency: measures the efficiency with which irrigated agriculture uses water resources in the production of crops and fibre.

Case studies in the Philippines and other Asian countries reveal that utmost benefits from irrigation development are not fully realized for a variety of reasons which leads to sub-optimal irrigation performance, as such, decrease in irrigated areas, cropping intensity, crop yield and among others [3]. Also, a number of studies conducted to assess the performance of several irrigation systems revealed that these systems performed below the expectations [4-9].

Lack of rainfall in the dry season and dry spells in the rainy season due to changing climate, however, are among the major constraints to rice production, and water productivity in paddy fields is perceived to be low. Improving the performance of irrigation scheme at MARIIS and CIS is an obvious issue for agricultural development. Irrigation efficiency, which is an indicator of effective water resources management, varies from area to area. A particular concern is water shortage within irrigation scheme command areas, particularly in the dry season or in dry spells during the rainy season [10].

In the case of MARIIS, another major concern is growing presence of fishponds constructed along the main and secondary canals in the upstream reach. Based on the result of a study, there were approximately 300 ha of fishponds already established along the south high canal area alone, whose water requirements are being drawn freely without any regulation. Further, the study revealed that about 6.73 m^3/s or equivalent to 28% of the 24 m^3/s irrigation diversion requirements (IDR) is lost due to excessive use of water by fishponds operators. On the other hand, Small Water Impounding Project (SWIP) is perceived to be performing poorly, which is the way below of what was expected based on the latest system inventory [11].

Therefore, improvement of efficiency can improve equity in water distribution and minimize the gap between potential crop water requirements and actual water use. In consequence, it will lead to the determination of the effectiveness of water use and the improvement of the livelihood of people [12]. Farmers can use lesser water or lower input investment while obtaining higher production and remaining more water in the sources, which can maintain the ecological cycle and environment of river basin.

Furthermore, in many irrigation projects, there is no baseline information (physical, institutional and management) regarding the levels of service to water users (farmers and fisherfolks) and the factors which affect those services. Establishing baseline information regarding the levels of service, determining standards and then determining how to meet them could be crucial for improving the design, upgrading and management of irrigation and drainage projects.

Hence, the objective of this research was to assess the performance of small and large reservoir irrigation schemes in Cagayan River Basin. Specifically, the paper aimed to determine and establish benchmarks for both productivity and performance using standard indicators—irrigation efficiency, adequacy of water supply, productivity of land and water resources and financial viability of the schemes.

2. Description of the Study Sites

The four systems (namely, small systems Divisoria, Garab, Lucban SWIP and large system NIA-MARIIS,

respectively) were all strategically situated within the Cagayan River Basin. The Cagayan River Basin lies in the Northeastern tip of the Philippines as shown in Fig. 1. The Cagayan River flows through the four mainland provinces and is the largest river system in the country. It is located between 15°52′ N-18°23′ N latitudes and 120°51′ E-122°19′ E longitudes. Based on the data from Ref. [13], the potential irrigable area in Cagayan Valley is about 472,640 ha, of which only 46.32% was irrigated as of the year 2000. That leaves more than 200,000 ha yet to be provided with irrigation facilities. These potential irrigable areas include rainfed areas and areas presently planted to corn and other agricultural commodities. While areas suitable for rice may have been underutilized, there are also marginal rice lands that are actually more suitable for other crops [14].

2.1 MARIIS at a Glance

The MARIIS has four distinct divisions I, II, III and IV as shown in Fig. 2. Pilot locations were considered as representation of typical canal system with appropriate

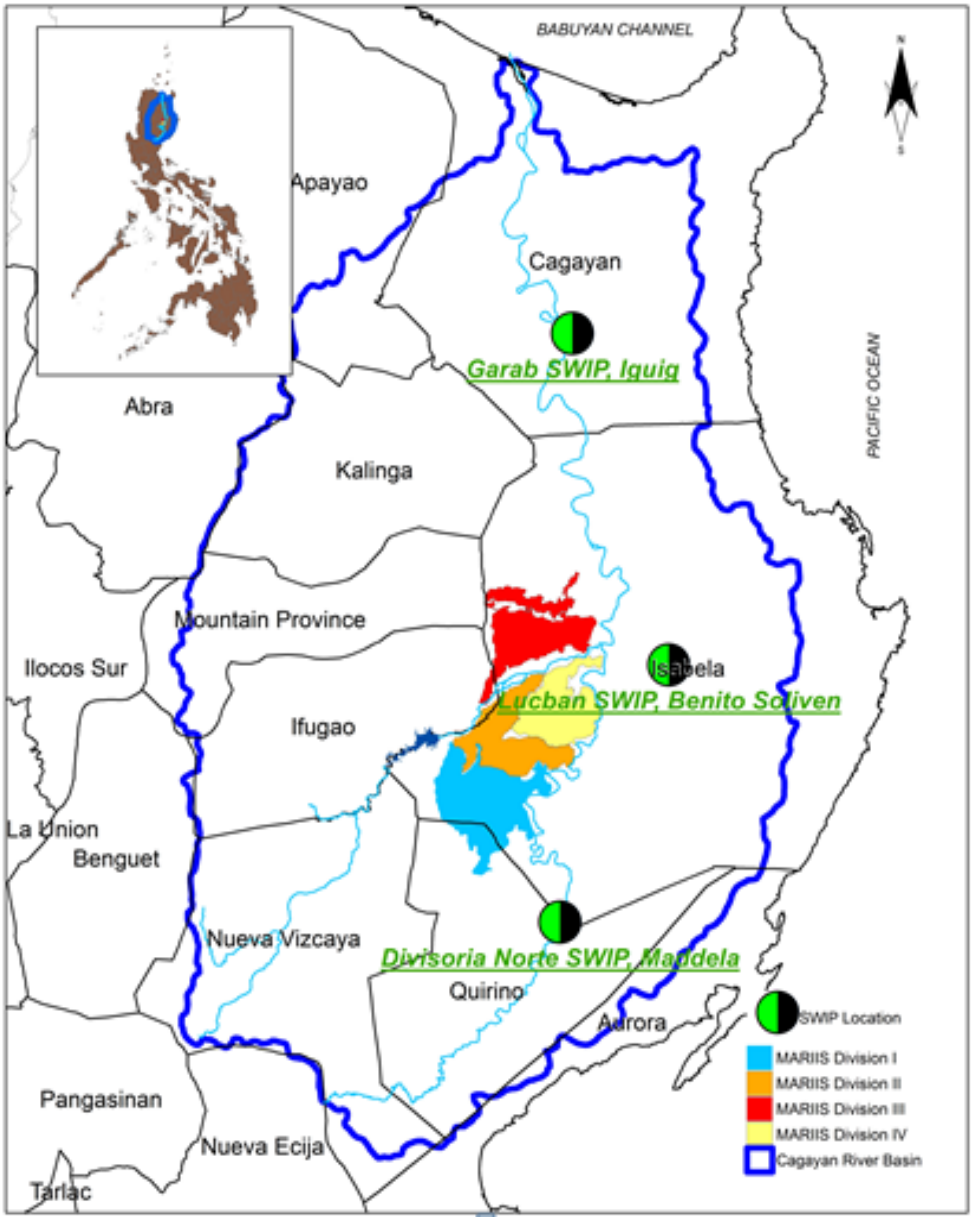

Fig. 1 Distribution of selected study sites.

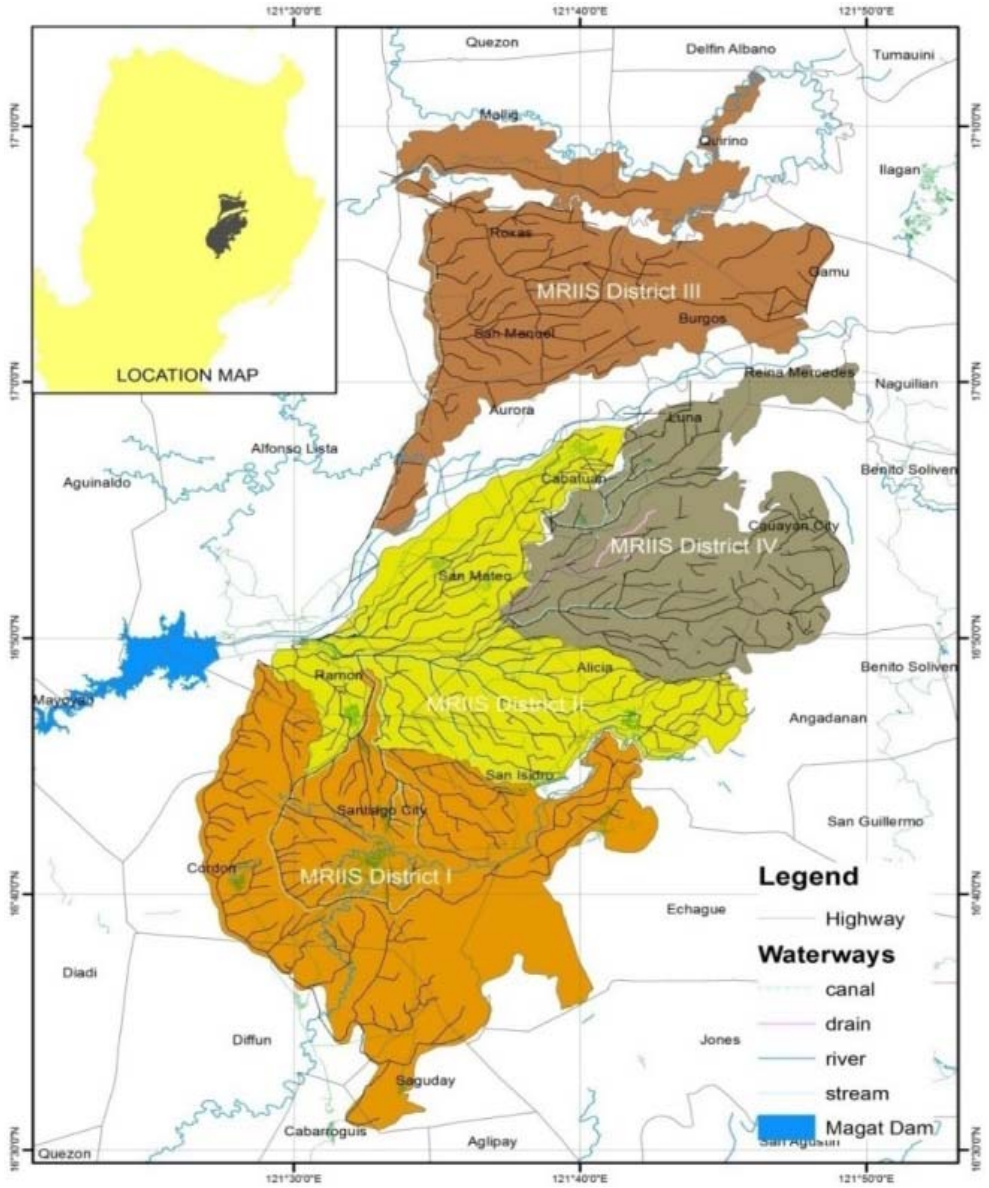

Fig. 2 MARIIS service area indicating the distinct divisions.

size of command areas, considering accessibility and availability of relevant information. Study period considered in the benchmarking analysis is five years covering two cropping season from 2008 to 2012.

The Magat River multi-purpose system was built in 1975 to provide dependable water supply for irrigation and power generation. The project area includes the service area of the two existing irrigation systems, namely MARIIS and Siffu Irrigation System (SIFRIS), and these together with all the appurtenant facilities and structures brought the present firmed-up service area to 84,795 ha. On the other hand, the Magat hydroelectric power plant constructed below the Magat dam has an initial installed capacity of 360 megawatts with a provision to increase its capacity to 540 megawatts. With the government's program to privatize the generating assets of the National Power Corporation (NPC) as mandated by the electric power industry reform act (EPIRA) law, the ownership of the power plant was transferred from the NPC to SN-ABOITIZ effective on April 25, 2007.

MARIIS 5-year (2008-2012) average service area is 78,496.20 ha, which composes of four distinct divisions, namely, division I (Santiago City cluster),

district II (San Mateo cluster), district III (Roxas cluster) and district IV (Cauayan cluster) with corresponding service area distribution 25.00%, 28.98%, 23.28% and 22.70%, respectively, as shown in Fig. 3.

2.2 Present Status of Irrigation Utilization

Besides of various measures taken so far to equalize the amount of targeted vs. actual cultivated area, there is still a gap between the two values as depicted in Fig. 4. A 5-year target and actual utilization within MARIIS from 2008 to 2012 is exhibited in Fig. 4.

Fig. 5 shows that there is an annual gradual improvement in terms of area served by the system from 2008-2012 by 3.34%, however, there is still under utilization of almost 2%. While in terms of 5-year season-wise representation of actual area irrigated as depicted in Fig. 5, remarkable linear increase of irrigated hectarage both during the dry and wet seasons were attained by 3.79% and 2.91%, respectively. Peak increase is noted for both seasons during the year 2011 with a slight decrease in irrigated area for the dry and wet seasons.

2.3 The Case of Small Water Impounding Projects in Cagayan River Basin

In Philippines, rainwater harvesting project (i.e., with its institutional title SWIP) is a structure constructed across a narrow depression or valley to hold back water and develop a reservoir that will store rainfall and run-off during the rainy season for immediate or future use.

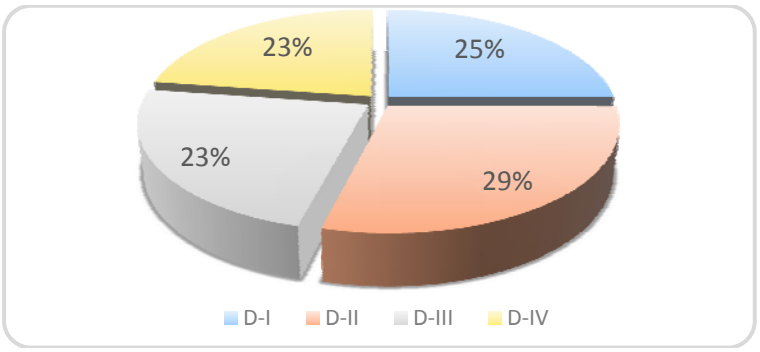

Fig. 3 MARIIS actual service area distribution.

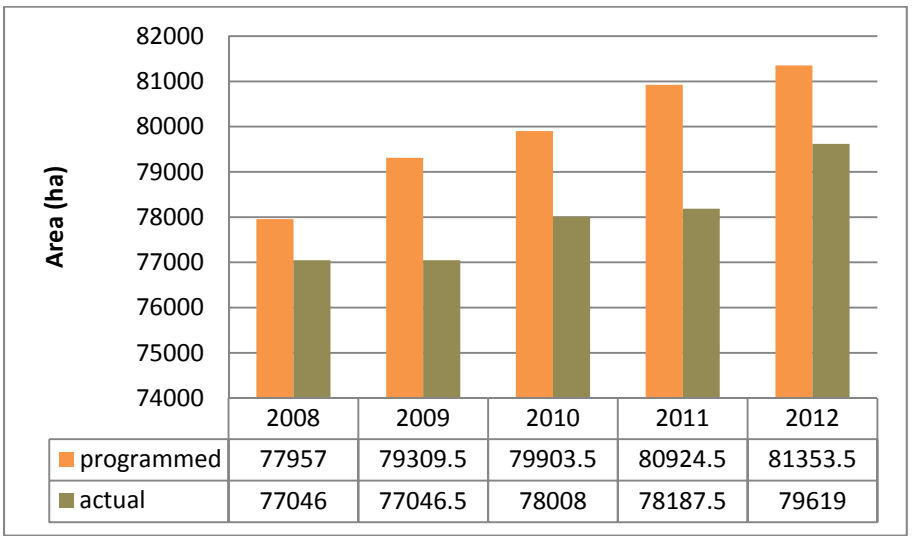

Fig. 4 MARIIS programmed vs. actual irrigated area.

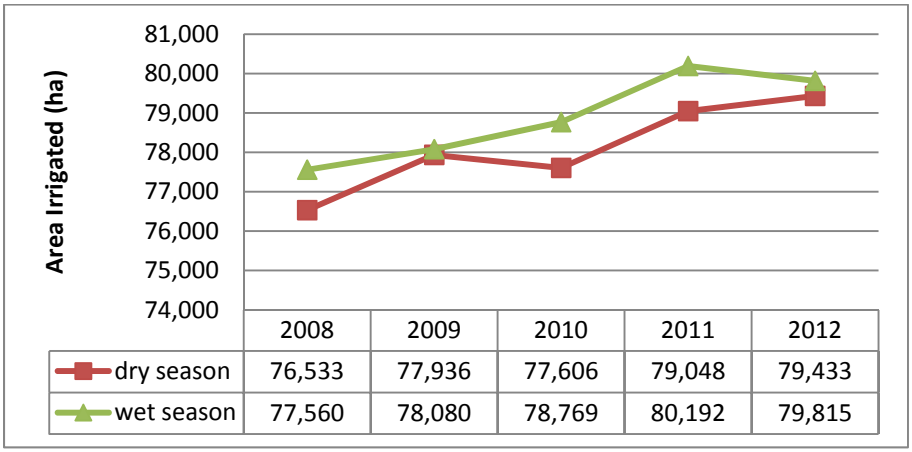

Fig. 5 MARIIS progress in irrigated hectarage.

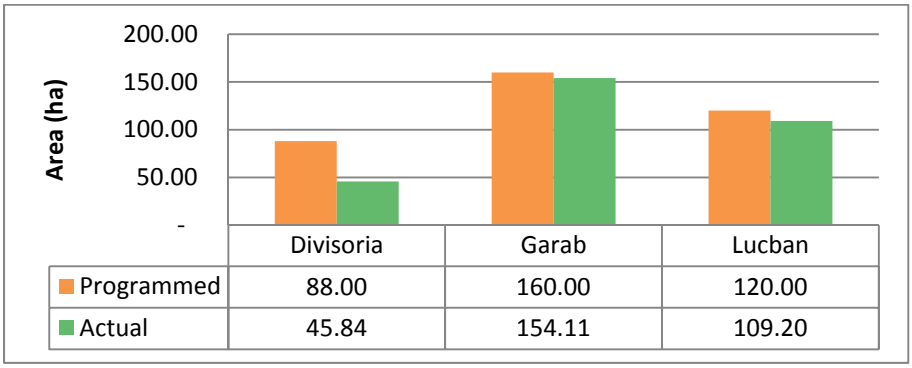

Fig. 6 SWIPs study area showing the programmed vs. actual area irrigated.

In December 2011, the Bureau of Soils and Water Management estimated that there are 103 units of SWIP in the Cagayan Valley region with a total service area of 6,353 ha and 4,929 farmer beneficiaries. However, there still is a gap between the programmed and actual irrigated area at selected sites, particularly at Divisoria SWIP with only 50% served by the system as depicted in Fig. 6.

3. Methods

3.1 Data Collection

General methods and activities were as follows: (1) review of documents and site validations; (2) key informant interviews, focus group discussions and household survey; (3) focus group discussions; (4) on-field measurements/inspections/observations.

3.2 Review of Documents and Site Validation

In the case of SWIPs, thorough study of previous reports, inventory, manuals and designs of SWIP in Cagayan Valley was conducted. Three SWIP pilot sites in Cagayan Valley were selected for detailed study. Initially, SWIP at Divisoria in Maddela Quirino, Garab in Ilagan Isabela and Iguig in Cagayan were selected and subjected to validation visit. The selection was made based on the earlier studies and recommendations by the Regional Agricultural Engineering Group (RAEG) of the Department of Agriculture. The identified SWIPs are considered ideal sites and representative sites of SWIPs in the region.

Existing and field data were collected related to water resources within the selected irrigation systems. Water discharges in various sections of main canals, cropped area for the two seasons and cropping intensity and paddy production.

3.3 Benchmarking Irrigation Systems Performance

The performance indicators used for this study was

adopted from the internationally recognized standard indicators set by IWMI, FAO, IPTRID and ICID. Such irrigation domains covered in the study are as follows:

(a) Service delivery: this domain includes two areas of service provision: (1) the adequacy with which the organization manages the operation of the irrigation delivery system to satisfy the water required and (2) the efficiency with which the organization uses resources to provide this service (financial performance);

(b) Productive efficiency: measures the efficiency with which irrigated agriculture uses water resources in the production of crops and fibre.

Table 1 shows the specific domains for benchmarking with their corresponding specific performance indicators selected.

4. Results

The results of the irrigation performance indicators analyses based on three domains—(1) system operation performance, (2) agricultural productivity and economics and (3) financial performance, respectively.

4.1 System Operation Performance

The provision of water for irrigation and electric generation are the purposes of the Magat dam reservoir system. However, the system's first priority is the provision of dependable irrigation water supply to farm lands. Though, distribution of such water resource is influenced by numerous factors, such as, physical, climatic, economic and other related factors which eventually affect the delivery performance of the system. Measurements on the system operation performance were based on specific indicators, such as, (a) delivery of the system, (b) annual irrigation water supply and (c) annual relative water supply. The results of system delivery efficiency, annual irrigation supply and relative water supply for MARIIS and the three SWIPs selected sites were presented in Fig. 7.

4.1.1 System Irrigation Efficiency

The system delivery efficiency was significantly different for the whole MARIIS, Lucban, Garab, and Divisoria schemes. The MARIIS shows the highest efficiency value of 59%, but Lucban SWIP is statistically comparable with efficiency value of 55%, while the two other SWIPs, namely, Garab and

Table 1 Specific domains used for benchmarking.

Domain	Performance indicator
Service/system operation performance	System irrigation efficiency Annual irrigation water delivery per unit irrigated area (m^3/ha) Annual relative water supply
Productive efficiency	Output per unit irrigated area (Peso/ha) Output per unit irrigation supply (Peso/m^3)
Financial performance	Cost recovery ratio Revenue collection performance Cost recovery ratio

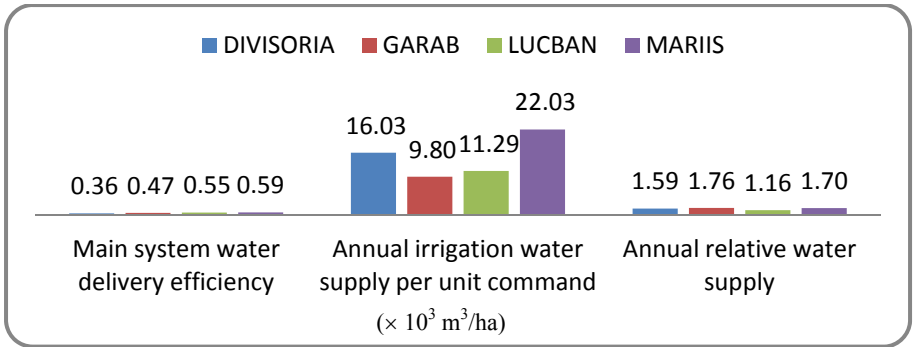

Fig. 7 System-wise operation performance indicators.

Divisoria were slightly lower than the previous systems with efficiency values of 47% and 36%, respectively. Although MARIIS and Lucban schemes are significantly higher compared to Garab and Divisoria SWIPs, these systems are quiet lower than irrigation efficiency of other irrigation schemes worldwide. Bandara [15] estimated that the irrigation efficiency of major irrigation system in Sri Lanka reach as high as 71%. Also, surface irrigation efficiency ranges between 50% and 60% in Israel, Japan and Taiwan [16].

4.1.2 Annual Irrigation Water Delivery per Unit Irrigated Area (m^3/ha)

This indicator is a measure on the total quantity of water supplied for irrigation throughout the year compared to the total area irrigated for the whole system selected. Although annual irrigation water supply per unit irrigated area depends on several factors, such as, water availability, cropping pattern climate, soil type, systems condition and management, the four systems average performance in terms of annual irrigation water delivery per unit irrigated area measured was significantly different as depicted in Fig. 7, where MARIIS registered the highest amount of 22,029.43 m^3/ha, tailed by Divisoria and Lucban SWIPs with comparable amount of 16,026.37 m^3/ha and 11,289.10 m^3/ha, respectively, and Garab registered the least amount of 9,795.96 m^3/ha.

4.1.3 Annual Relative Water Supply

The relative water supply is a suitable indicator to show whether crop water requirements of an area were sufficiently provided. Annual relative water supply for all the systems considered was comparable as reflected in Fig. 7. However, values are relatively lower as compared to the schemes of other systems in the world with only 1.70, 1.16, 1.76 and 1.59 for MARIIS, Lucban, Garab and Lucban SWIP, respectively, while that of the 18 irrigation systems located in 11 countries in the Asian region vary between 0.8 and 4.0, with more a half of these systems have annual relative water supply of greater than 2 [17, 18]. These low values within the selected systems indicate that adequacy of supplied irrigation water is adversely affected by the low system delivery efficiencies.

4.2 Agricultural Productivity and Economics

4.2.1 Output per Unit Irrigation Supply (kg/m^3)

This water productivity indicator is a measure of the optimal utilization of water in relation to the total agricultural production served by the system. The comparison of data on output per unit irrigation supplied is shown in Fig. 8. Although differences are not pronounced between Divisoria, Garab and MARIIS systems, however, Lucban SWIP has significant output value of 0.35 kg/m^3. According to the water productivity categorization levels [19], the systems performance ranks as low if less than 0.35 kg/m^3, moderate if between 0.3 kg/m^3 to 0.4 kg/m^3 and high if greater than 0.4 kg/m^3. Accordingly, the

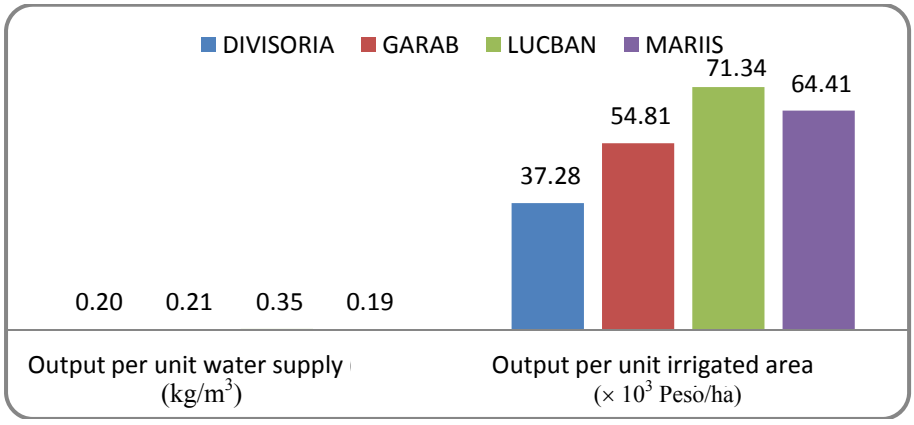

Fig. 8 System-wise agricultural productivity performance indicators.

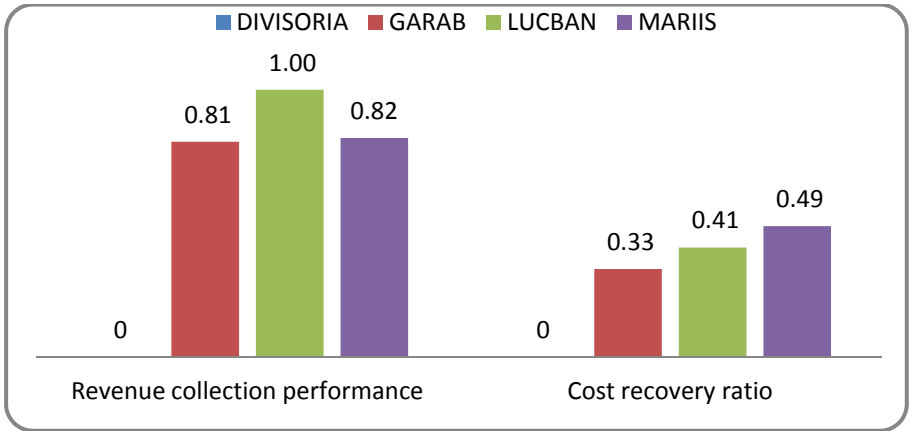

Fig. 9 System-wise financial performance indicators.

productivity classification of Lucban SWIP can be considered as moderate; while the rest Divisoria, Garab and even MARIIS system are categorized under low productivity index. Subsequently, besides Lucban was considered to be the highest among the four irrigation systems under study, its grain productivity was still lower compared to global grain average ranging from 0.76 kg/m^3 to 1.23 kg/m^3 [20].

4.2.2 Output per Unit Irrigated Area (Peso/ha)

This indicator quantifies the performance of the system in terms of production output in a given unit irrigated area in Peso/ha basis. This indicator is very much important, since water is the only factor on which the service provider has full control linked with the adoption of improved/latest technology, as the population grows while land holding per capita goes on reducing. For the four systems, land productivity performance is reflected in Fig. 8, where Lucban yielded the highest output with comparable output value of MARIIS of 71,336.22 Peso/ha and 64,412.15 Peso/ha, respectively. Generally, results indicate a highly significant scope to increase land and water productivity for the four irrigation systems as indicated by the variable and low productivity performance.

4.3 Financial Performance

4.3.1 Revenue/m^3 of Irrigation Water Supply (Peso/m^3)

Revenue/m^3 of irrigation water supply is the ratio of the total revenue and gross volume of water supplied for irrigation during the irrigation year. This indicator is very important measure, as every drop of water needs to be used efficiently and economically. In the case of Lucban, irrigation fee collection performance is 100%; while 81% and 82% collection efficiency were attained at Garab and MARIIS, respectively, as shown in Fig. 9. However, there was no collection effort made at Divisoria SWIP.

4.3.2 Cost Recovery Ratio

Cost recovery ratio is the ratio of recovery of water charges to the cost of providing the service. It is imperative to consider this indicator for the design of water rates and recovery mechanism for the sustainable operation of the system. On the basis of sustainability, the theoretical cost recovery ratio for any system should be at least equal to one. However, the four systems have poor cost recovery ratio of 0, 0.33, 0.41 and 0.49 for Divisoria, Garab, Lucban and MARIIS, respectively, as presented in Fig. 9, where it is half-way below the theoretical value for system sustainability [21].

5. Conclusions

This study focused on assessment of the performance of small (Lucban, Garab and Divisoria SWIPs) and large (NIA-MARIIS) reservoir irrigation schemes. The results showed relatively low performance of the four systems being assessed as manifested by irrigation efficiency of 59%, 55%, 47%

and 46% for MARIIS, Lucban, Garab, and Divisoria, respectively. Thereby, improving the systems delivery and distribution efficiency could eventually support the targeted service area of each system as being indicated by the considerable amount annual relative water supply of 1.70, 1.16, 1.76 and 1.59 for MARIIS, Lucban, Garab and Lucban SWIP, respectively, which is quite near the world average relative irrigation water supply of 2.0.

In terms of water and land productivity of the four scheme, it was 0.20, 0.21, 0.35, 0.19 kg/m^3 and 37,276.01, 54,813.40, 71,336.22, 64,412.15 Peso/ha, respectively, for Divisoria, Garab, Lucban and MARIIS schemes. Besides, the low productivity of the systems, except for Lucban, were classified as moderate performing, but quit low compared to global average productivity index ranging from 0.76 to 1.23 kg/m^3.

Financial sustainability of the systems were extremely poor as indicated by the low cost recovery ratio of 0, 0.33, 0.41 and 0.49 for Divisoria, Garab, Lucban and MARIIS schemes, respectively, which were exceptionally below the standard value of at least one. Thus, to insure sustainable operation of the systems, wise mechanism recovery design should be implemented for each system being considered.

The study confirmed that a national and communal irrigation schemes within the Cagayan River Basin were on the average performed similarly; however, both schemes performed quit below the world standard in terms of irrigation efficiency, land and water productivity levels and financial viability of the schemes. With the result of the analysis, it is therefore necessary to consider an over-all system improvement in terms of the three basic domains used, as such, (1) system operation performance, which includes but not limited to improvement on conveyance and distribution systems; (2) agricultural productivity and economics, which embraces superior production management practices, like adoption of high yielding varieties, appropriate fertilization technologies, water management scheme, etc.; (3) financial performance, which should consider wise mechanism recovery design and implementation.

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Karyotype Analysis of the Fiddleneck (*Phacelia tanacetifolia* Benth.)

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Abstract: Two varieties of fiddleneck (*Phacelia tanacetifolia* Benth.) plant were determined for visualizing somatic chromosomes. The 4-5 days old root tips were pre-treated in 6% α-monobromonaphtalane in +4 °C for 7.5 h, then fixed in glacial acetic acid for 30 min and transferred to 70% ethanol for long storage. When the root tips were analyzed, they were hydrolyzed with 1 N HCl for 13 min at room temperature (25 °C). After hydrolyzing, root tips were stained with 2% aceto orcein in darkness for 2.5 h. The squash method for preparation was used for chromosomal investigations. The chromosome length (C), relative length (RL), the long arm (L) and short arm (S) lengths, arm ratio (AR; L/S) and centromeric index (S/C) were calculated for caryologic parameters. The ideograms and detailed chromosome morphology measurements of the species were performed by the use of MicroMeasure 3.3. According to results, fiddleneck (*Phacelia tanacetifolia* Benth.) has $2n = 22$ chromosomes and the karyotype formulas of two varieties of fiddleneck were 16 median and 6 submedian (16 m + 6 sm).

Key words: Fiddleneck, cytogenetic, karyotype, micro measure, chromosome number, *Phacelia tanacetifolia*.

1. Introduction

Fiddleneck (*Phacelia tanacetifolia* Benth.) is a genus of about 200 species of annual or perennial herbaceous plants, which are members of the family Boraginaceae and are native to Southwestern United States and Mexico [1]. Many species can be cultivated as honey plants and garden plants due to its aesthetic appearance. Fiddleneck (*Phacelia tanacetifolia* Benth.) is an annual plant. Moreover, the genus is one of the top 20 most common bee plants as a rich source of nectar and pollen, most preferred by honey bees [2]. It is particularly an important source of nectar for wild bees, such as *Bombus* [3]. Since fiddleneck (*Phacelia tanacetifolia* Benth.) is attractive to a large number of insects, it can be used as a trap plant in the field as a biological preventive against the harms of crop plants [4]. Thus, it can also help the environment by minimizing the application of pesticides [5]. It is also attractive to hoverflies (family Syrphidae), which are useful as biological pest control agents, because they feed on aphids and other pests [6]. The photodormant seeds of fiddleneck (*Phacelia tanacetifolia* Benth.) can only germinate in the dark [7].

Two varieties of fiddleneck (*Phacelia tanacetifolia* Benth.), which is used as materials in this study, is registered officially in Turkey. Also, these varieties are very important for bee forage and beekeeping. In addition, these two varieties of fiddleneck are thought to use as parental plant in breeding programmes. After determining chromosome numbers and caryological features of these varieties, it is planned to intercross with their relatives which have same ploidy level. Although, there has been a lack of cytological information in the relevant literature in regard to the subject of the study—fiddleneck (*Phacelia tanacetifolia* Benth.) and no caryological studies have been reported. Therefore, the study aimed to determine the number of chromosomes, cytological characteristics and the methods that can be utilized to characterize the genus of fiddleneck (*Phacelia tanacetifolia* Benth.).

2. Materials and Methods

The seeds of fiddleneck (*Phacelia tanacetifolia*

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Benth.) in the karyotype analysis were obtained from Saglamtimur Aegean Agricultural Research Institute, and the type of Enton was provided by the gene bank.

For visualizing somatic chromosomes, root tips were obtained from germinated fiddleneck (*Phacelia tanacetifolia* Benth.) seeds germinated in petri dishes at room temperature (25 °C). The 4-5 days old root tips were pre-treated in 6% α-monobromonaphtalane in +4 °C for 7.5 h, then fixed in glacial acetic acid for 30 min and transferred to 70% ethanol for long storage. When the root tips were analyzed, they were hydrolyzed with 1 N HCl for 13 min at room temperature (25 °C). After hydrolyzing, root tips stained with 2% aceto orcein in darkness for 2.5 h. Then, finally they were squashed in 45% acetic acid. Slides were observed with Olympus BX-51 microscope, photographs were taken with Olympus BX-51 camera at room temperature (25 °C) and the magnification was 8,000×. Six chromosomal parameters were measured by MicroMeasure 3.3 program [8], i.e., chromosome length (C), relative length (RL), the long arm (L) and short arm (S) lengths, arm ratio (AR; L/S), centromeric index (S/C). Ideograms were drawn based on long arm length/short arm length. Karyotype formulas of two varieties of fiddleneck were determined by the methods in Ref. [9].

3. Results and Discussion

In this study, the somatic chromosomes number and cytological features of two varieties of fiddleneck (*Phacelia tanacetifolia* Benth.) were determined. Table 1 presents the caryological characteristics on the cultivars of Enton and Saglamtimur. The images of two fiddleneck (*Phacelia tanacetifolia* Benth.) varieties and

Table 1 Caryological features of Enton and Saglamtimur cultivars.

Number	Type	Chromosome length (μm)	Relative length (%)	Long arm lenght (μm)	Short arm lenght (μm)	Arm ratio	Centromeric index
Enton cultivar							
1	m	4.73	11.42	2.80	1.93	1.45	0.81
2	sm	4.26	10.27	2.87	1.39	2.06	0.65
3	m	4.13	9.97	2.48	1.65	1.50	0.80
4	m	3.97	9.59	2.36	1.62	1.46	0.81
5	sm	3.85	9.30	2.45	1.40	1.75	0.73
6	m	3.70	8.93	1.92	1.78	1.08	0.96
7	m	3.56	8.59	2.20	1.36	1.62	0.76
8	m	3.49	8.43	2.07	1.43	1.45	0.82
9	m	3.43	8.27	1.94	1.49	1.30	0.87
10	sm	3.36	8.12	2.27	1.10	2.50	0.64
11	m	2.94	7.11	1.75	1.19	1.47	0.80
Total		41.42					
Saglamtimur cultivar							
1	sm	5.79	13.15	3.73	2.06	1.81	0.72
2	sm	4.81	10.92	3.08	1.73	1.78	0.72
3	sm	4.65	10.56	3.00	1.65	1.82	0.71
4	m	4.50	10.22	2.77	1.73	1.60	0.77
5	m	4.29	9.75	2.59	1.71	1.51	0.80
6	m	3.98	9.04	2.13	1.84	1.16	0.93
7	m	3.51	7.97	2.01	1.42	1.42	0.81
8	m	3.37	7.66	1.99	1.39	1.43	0.82
9	m	3.26	7.41	1.77	1.49	1.19	0.91
10	m	3.12	7.10	1.76	1.36	1.29	0.87
11	m	2.74	6.23	1.85	0.89	2.08	0.65
Total		44.01					

m: median; sm: sub-median.

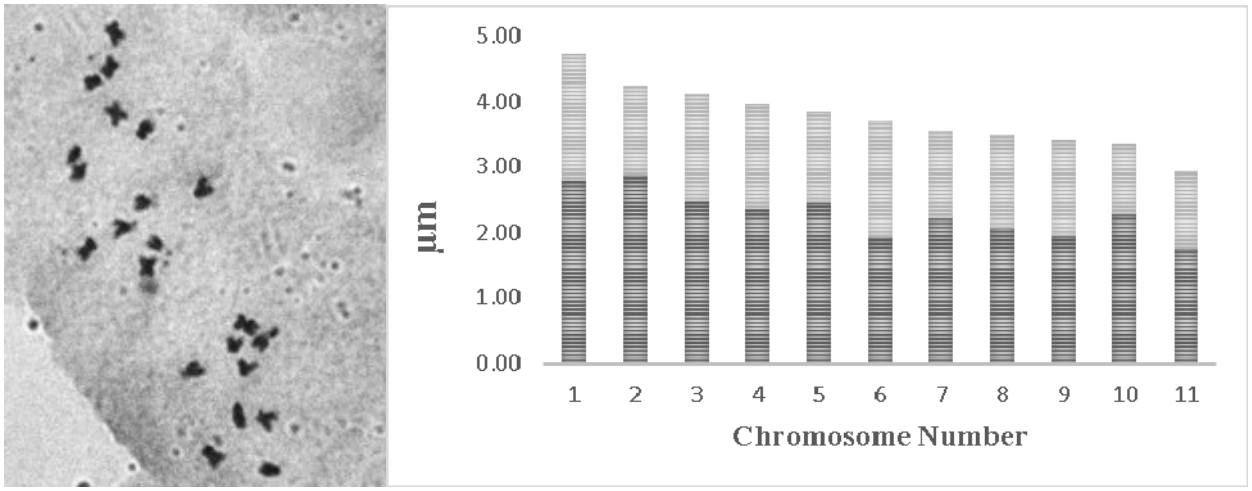

Fig. 1 Somatic metaphases and ideogram of Enton cultivar.

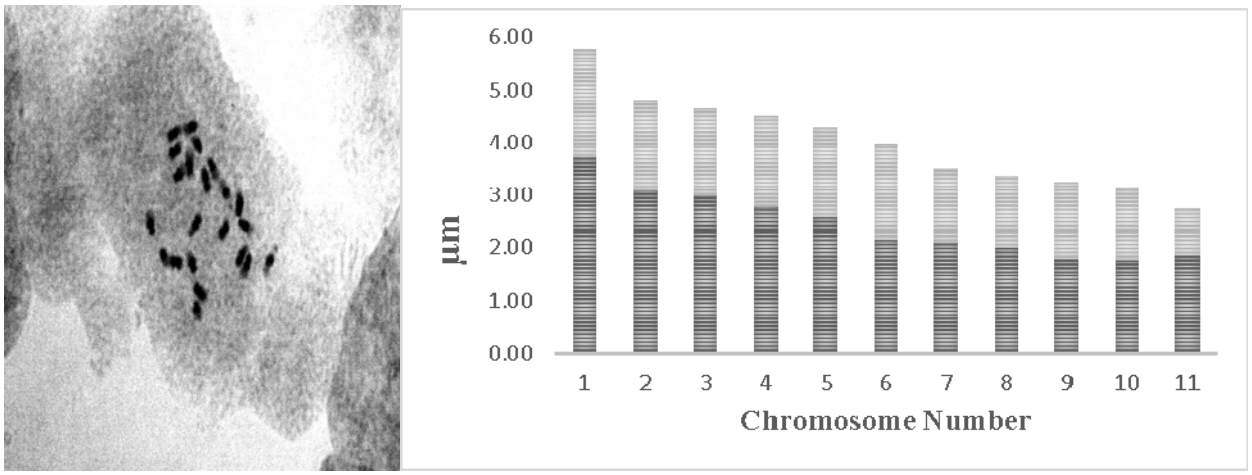

Fig. 2 Somatic metaphases and ideogram of Saglamtimur cultivar.

ideograms were shown in Figs. 1 and 2. The results indicated that the chromosome number of fiddleneck (*Phacelia tanacetifolia* Benth.) is $2n = 22$. These results are consistent with the results obtained by Sugiura [10] and Cave and Constance [11].

Saglamtimur cultivar has the maximum and minimum values for six parameters. There are the smallest and biggest chromosome length (2.74 µm and 5.79 µm, respectively), the lowest and highest relative length (6.23% and 13.15%, respectively), the maximum long arm length (3.73 µm) and the minimum short arm length (0.89 µm). Enton cultivar has the smallest and biggest arm ratio (1.08 and 2.50, respectively). In terms of the centromeric index, Enton cultivar has the smallest and biggest centromeric index (0.65 and 0.96, respectively).

The karyotype formulas of the species of fiddleneck (*Phacelia tanacetifolia* Benth.), on which the karyotype analysis was carried out, were found identical $2n = 22$ (16 median + 6 submedian).

3.1 Enton Cultivar

According to results of Enton cultivar (Table 1), the biggest chromosome length was calculated 4.73 µm, and the smallest chromosome length was calculated 2.94 µm. The highest relative length value was measured 11.42% and the smallest relative value was measured 7.11%. Long arm length was measured between 2.87 µm and 1.75 µm, while short arm length was measured between 1.93 µm and 1.10 µm. Chromosomes arm ratios were calculated between 1.08 and 2.50. The centromeric index was measured

between 0.96 and 0.64.

3.2 Saglamtimur Cultivar

As a results of Saglamtimur cultivar (Table 1), the largest chromosome length was calculated 5.79 μm, while the smallest chromosome length was calculated 2.74 μm. The largest relative length value was measured 13.15% and the smallest relative value was measured 6.23%. While long arm length was measured between 3.73 μm and 1.76 μm, short arm length was measured between 2.06 μm and 0.89 μm. Chromosomes arm ratios were between 2.08 and 1.16. The centromeric index varies between 0.93 and 0.65.

4. Conclusions

Turkey has the remarkable place for bee culture in the world. The plants like fiddleneck (*Phacelia tanacetifolia* Benth.), which has better quality, should evaluate more in bee forage for making better situation about bee industry in Turkey. As a result in this research, the varieties of fiddleneck has $2n = 22$ chromosomes and the karyotype formulas is 16 median and 6 submedian, so they can be utilized as parental plant, because of determined chromosome numbers, caryomorphological features and ploidy level. Additionally, this research will make opportunity to investigate the origin of varieties of fiddleneck (*Phacelia tanacetifolia* Benth.) and then new varieties of fiddlencek are came into use for bee forage farmers in Turkey and the world.

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Autumn Cultivation of Farewell-To-Spring (*Clarkia amoena* A. Nelson & J. F. Macbr.) in Unheated Foil Tunnel in Lower Silesia Condition

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Abstract: In conditions of Poland, farewell-to-spring never was cultivated for cut flowers in unheated foil tunnel. Specific conditions of Lower Silesia, together with the short cultivation cycle and small demands of this plant, are promised for its cultivation in this region of Europe. The aim of the study was to evaluate the usefulness of two farewell-to-spring varieties for the cultivation for cut flowers in unheated foil tunnel in Lower Silesia conditions during autumn time. The two-factorial experiment was carried out by the method of random blocks in Research-Development Station of Vegetable and Ornamental Plants of Wrocław University of Environmental and Life Sciences. The first factor was the variety—"Grace White" and "Brillant" and the second was the year of cultivation—2012 and 2013. Measurements included number and length of flower stems, number of flower buds and leaves per flower stem, fresh and dry weight of flower stem and leaves color parameters. Total chlorophyll content was also determined. Studies showed that autumn cultivation of farewell-to-spring is reasonable because of its high yield (about 640 flower stems/m^2) with long flower stems from 50 cm up to 80 cm. In Lower Silesia conditions, the variety "Grace White" has proven to be better; it produced higher number of flower stems with higher number of lowers buds and thus flowers. In terms of thermal conditions, more favorable was year 2013, in which the plants produced longer flower stems and had higher total chlorophyll content in leaves. After inserting the flower stems in tap water, all flower buds developed.

Key words: Farewell-to-spring, autumn cultivation, foil tunnel, cut flowers, Lower Silesia.

1. Introduction

Farewell-to-spring (*Clarkia amoena* (Lehm.) A. Nelson & J. F. Macbr.) occurs naturally in the Southwestern United States in the area of California [1]. Sometimes, it is commonly called in Poland as summer azalea, because its flowers resemble potted azaleas. Other common names are godetia or satin flower [2]. The plant is cultivated as annual and used for flowerbeds decoration. In Poland, it is cultivated occasionally for cut flowers in the ground, but it remains very popular in Italy and Israel. In warm states of North America, it can be grown throughout the year. The best condition for its growth is dry climate with low temperature, thanks to their flowering that can be controlled [3].

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In Poland, interest in energy-saving production increases due to high heating costs. One of the most important alternatives is searching for cultivation of attractive ornamental plants with small thermal requirements. Farewell-to-spring cultivated for cut flowers in unheated foil tunnel can be included to group of such plants. In climate conditions of Poland, *Chrysanthemum*, sweet peas, *Alstroemeria* and *Dianthus* are commonly grown for cut flowers [4]. In Western Europe, there have also been developed efficient methods of cultivation of ornamental plants, such as Persian cyclamen, true oxlip and dusty miller [5].

In Poland, farewell-to-spring has never been cultivated in unheated foil tunnel for cut flowers before. Specific conditions of Lower Silesia are promised for its cultivation and give a chance to increase the assortment of cut flowers on Polish market during autumn season. Short production cycle

and low thermal demands of this plant prompted the authors of this research to evaluate the usefulness of two farewell-to-spring varieties—"Grace White" with white flowers and "Brillant" with single carmine-pink flowers.

2. Materials and Methods

The two-factorial experiment was set up by the method of random blocks in Research-Development Station of Vegetable and Ornamental Plants of Wrocław University of Environmental and Life Sciences. The first factor was the variety of farewell-to spring—"Grace White" and "Brillant" (W. Legutko, Poland). The second factor was year of cultivation; experiment was carried out in 2012 and repeated with the same method in 2013. Seeds were sown to multi-pots in peat substrate in early July. In early August, seedlings size of 12-15 cm were planted on the ground ridges under unheated foil tunnel with dimensions 9 m × 30 m, spacing 25 cm ×15 cm on plots 5 m × 2 m. Each variety was planted in four replications (plots) for 30 plants. Soil on ridges belongs to class IIIa (good arable land complex), ranked as degraded light clays, which was enriched with peat substrate (Klasmann, Germany) (at dose 200 dm^3/plot) in order to loosen its structure. The organic matter content was at level of 2.8%, the pH (determined in distilled water at a volume ratio of 2:1 water to soil) was 8.2 and the salinity 155 μS/cm. The nutrient content was 55 mg/dm^3 N-NO_3^-, 37 mg/dm^3 P, 138 mg/dm^3 Mg, 125 mg/dm^3 K and 4,800 mg/dm^3 Ca. In late August, plants were top dressed with Yara Milla complex fertilizer, which was composed of 12% N (including 5% N-NO_3^- and 7% N-NH_4), 11% P-P_2O_5, 18% K-K_2O, 2.7% Mg-MgO, 8% S, 0.015% B, 0.2% Fe, 0.02% Mn and 0.02% Zn at a dose of 3 g/plant. After 13 weeks (in the first decade of November), in the phase of one developed flowers number and length of flower stems, flower buds and number of leaves were measured and total chlorophyll content and leaves color parameters were determined. Then half of the plants from each replication (15) were collected to determine the fresh and dry weight of flower stems, the other half was collected for cut flower. After inserting flower stems in the tap water, the ability of flower buds to develop into flowers was evaluated.

The color of the leaves was determined in fresh leaves, just after collection with a HunterLab MiniScan EZ colorimeter in reflected light in the range of 400-700 nm using a scale of CIE L*a*b*. The parameter L* (brightness) has a value from 0 (black) to 100 (white). For the parameter a* (tone color), the positive determines the amount of red color, while the negative stands for green color. For the parameter b* (tone color), the positive determines the amount of yellow color, while the negative is blue color amount. Chlorophyll content was determined in fresh weight of leaves using spectrophotometric method.

The data were subjected to the analysis of variance (ANOVA). The *F*-test was used to identify the treatments main effects and interactions followed by Tukey's range test at the 0.05 significance level.

Lower Silesia is the region of Poland with the longest vegetation period that lasts 226 d. The longest thermal season in summer lasts 92 d, starting from July 1st. Autumn starts at September 1st and lasts 68 d. Wrocław is the warmest city in Poland with average year air temperature exceeding 8.7 °C. Moreover, urban heat island effect is very pronounced, where the increase in annual mean temperature is about 1.5 °C and the mean monthly temperatures reach 3-7 °C as compared to suburban areas [6, 7]. The temperature curve during cultivation of farewell-to spring is shown in Table 1.

3. Results and Discussion

Cultivation of farewell-to-spring in conditions of Lower Silesia in unheated foil tunnel is possible. The "Grace White" variety produced more flower stems in comparison to "Brillant". Research conducted by

Table 1 Mean air temperature (°C) during cultivation of farewell-to-spring for cut flowers in unheated foil tunnel.

Year	Temperature in different months				
	July	August	September	October	November
2012	20.1	19.7	14.8	5.6	0
2013	20.0	21.0	13.8	11.6	5.8
Long-term average 1991-2000	18.9	18.7	14.0	9.0	3.4

Table 2 Mean values of selected morphological features of farewell-to-spring.

Variety	Year		Mean
	2012	2013	
Number of flower stems			
"Grace White"	23.92[b]	28.60[a]	26.27[a]
"Brillant"	24.32[b]	20.75[b]	22.54[b]
Mean	24.13	24.68	
Length of flower stems (cm)			
"Grace White"	53.08[b]	73.07	63.39
"Brillant"	57.35[b]	80.25[a]	68.80
Mean	55.21[b]	76.98[a]	
Number of flower buds per flower stem			
"Grace White"	15.50[b]	20.25[a]	17.87[a]
"Brillant"	14.25[b]	15.50[b]	14.87[b]
Mean	14.89[b]	17.89[a]	
Number of leaves per flower stem			
"Grace White"	89	95	92
"Brillant"	90	92	91
Mean	90	94	

[a, b]Means with different letters are statistically different.

Janowska [8] also indicates that various varieties of carnation may differ in number of flower stems. In this study, a factor—the year of cultivation had no effect on the number of flower stems of farewell-to-spring. Taking into account factors interaction, most flower stems were produced by "Grace White" variety in the second year of study, while "Grace White" in 2012 and "Brillant" in both years of cultivation resulted in the least (Table 2). In case of carnation, different numbers of flower stems of varieties were obtained in subsequent years of research. The variety "Rapid Weiss" produced the most flowers stems in the first year of cultivation, while "Heitmatland" and "Pink Beauty" varieties yielded better in the second year of cultivation [8].

In 2012 both varieties produced shorter flower stems and in 2013 flower stems length increased almost 40%, comparing to the previous year. Taking into account the factors interaction, in 2012 both varieties "Grace White" and "Brillant" were statistically the lowest, while in 2013 the highest was farewell-to-spring "Brillant" variety (Table 2). Analyzing farewell-to-spring flower stems length in relation to the tested varieties, it was found that there was no effect on this characteristic. In studies of Janowska [8], also no significant difference in length was found between the flower stems of carnation varieties grown in unheated foil tunnel.

More flower buds formed plants in the variety "Grace White". In 2013 both varieties of farewell-to-spring produced more flower buds than in 2012. The greatest number of flower buds was observed in "Grace White" variety in 2013. On average, "Grace White" variety had the least of flower buds in 2012 and "Brillant" had the least flower buds in both years of cultivation (Table 2). It can be seen that the number of flower

buds was closely related to the number of flower stems. Obtained results were confirmed by Czekalski and Czemplik [9] in their research of farewell-to-spring cultivation for cut flowers in ridges on open ground. In this case, the variety, which produced the most of side shoots and thus had the most flower buds, was "Karminowa" variety.

In 2012, "Grace White" variety started flowering on November 8th, while "Brillant" did on November 10th. In 2013, both varieties started flowering earlier than previous year, on November 1st and on November 5th, respectively. It might be caused by temperature curve during cultivation. Optimum temperature for farewell-to-spring is 15-18 °C [3], and in October and November of 2013, during flower buds setting and flowers development, the temperature was higher (Table 1).

Flower stems were collected in phase of one developed flower, but ultimately after placing in water all flower buds developed into flowers. These results are confirmed by Czekalski and Czemplik [9]. After placing flowers shoots in water, regardless of its type and the addition of conditioner, all flower buds developed. Also, the research of Anderson [10] showed that all flower buds opened to normal size and color, moreover, performance in tap water was superior to performance in a commercial preservative solution. These results highlight the usefulness of farewell-to-spring for cut flowers production not only because of the opening up of all flower buds, but also due to its small requirements.

On the basis of the plants density and the number of

Table 3 Mean values of fresh and dry weight of flower stems, total chlorophyll content and color parameters of leaves.

Variety	Year		Mean
	2012	2013	
Fresh weight of flower stem (g)			
"Grace White"	11.70	12.60	12.15
"Brillant"	11.80	12.40	12.10
Mean	11.75	12.50	
Dry weight of flower stem (g)			
"Grace White"	5.2	5.6	5.4
"Brillant"	5.0	5.4	5.2
Mean	5.1	5.5	
Total chlorophyll content (mg/g of fresh weight)			
"Grace White"	0.806	0.934	0.870
"Brillant"	0.805	0.921	0.863
Mean	0.806[b]	0.928[a]	
Brightness L*			
"Grace White"	32.88	35.88	34.38
"Brillant"	33.43	34.46	33.95
Mean	33.16[b]	35.17[a]	
Tone color a*			
"Grace White"	-8.81	-9.31	-9.06
"Brillant"	-8.86	-8.99	-8.93
Mean	-8.84	-9.15	
Tone color b*			
"Grace White"	12.07	12.61	12.34
"Brillant"	12.26	12.54	12.40
Mean	12.17[b]	12.58[a]	

[a, b]Means with different letters are statistically different.

flower stems per single plant, the yield of flower stems/m^2 can be estimated. At a density ratio of 26.7 plants and harvest an average of 24 stems/plant, yield of flower stems will be shaped to an average of 640.8 stems. The obtained result is satisfactory. In the experiment conducted by Topa and Tallarico [11] in Italy in the spring cultivation of farewell-to-spring from one plant, they achieved an average yield of 5.6 stems/plant, which give 112 flower stems/m^2. In addition, 88% of collected stems were classified as class I (stem length of 50-55 cm), while in this research all plants exceed height of 50 cm even in the first year of study, in which both varieties were statistically the lowest.

None of the factors nor their interaction had influence on number of leaves of per flower stem (Table 2), as well as on fresh and dry weight of flower stems (Table 3). Total chlorophyll content was determined only by the year of cultivation and was higher in 2013 (Table 3). It might be associated with temperature during measurements; in November 2012, average temperature was 0 °C and in low temperatures chlorophyll breaks down. Taking into account the color parameters of leaves, the year of cultivation influenced brightness of leaves and tone color b*. In 2013 leaves were brighter and had more yellow tone color, while in 2012 they were darker and less yellow. None of the factors nor their interaction had influence on tone color a*.

There are many indications that farewell-to-spring could become a popular cut flower in Poland. Temperature curve in Lower Silesia during autumn cultivation is close to optimum and allows to obtain high yield of long flower stems. To make cultivation of this plant beneficial for polish producers, more acceptance from consumers is needed, which will influence the demands for this plant and its availability on the Polish market.

4. Conclusions

Autumn cultivation of farewell-to-spring is reasonable because of its high yield of long flower stems. Variety that produced higher number of flower stems with higher number of lowers buds and thus flowers was “Grace White”. In 2013, plants produced longer flower stems, had higher total chlorophyll content in leaves, but leaves were brightener and had more yellow tone color than previous year. After inserting the flower stems in tap water, all flower buds developed.

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First Report of an Outbreak of Contagious Ecthyma in Camels (*Camelus dromedarius* and *Camelus bactrianus*) in Iran

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Abstract: Camel contagious ecthyma (CCE) has been reported in Mongolia, Kenya, Somalia and Sudan. CCE is caused by a *Parapoxvirus* affecting young animals by producing lesions around the lips and nostrils. The generalized form of CCE is uncommon. The aim of this study was to submit the first report of contagious ecthyma in Iran and also to help clinician to diagnose this disease with heavy economic losses easier. In the paper, an outbreak of CCE in dromedary and bactrian camels in Qom province of Iran was described and clinical signs were observed in one camel herd in October 2009. Nodules and scabs from seven affected animals were collected for virus identification. Total extracted DNA was used for polymerase chain reaction (PCR) to amplify a fragment of *Parapoxvirus B2L* gene. Results showed that camel calves (n = 27) less than one year old and one male bactrian camel were affected (no adult female camels were found to be infected). The prevalence of the disease in the herd, adult camels and camel calves was 30.33%, 1.5% and 100%, respectively. Affected animals showed the swelling of head with nodular lesion around the lips. It then developed to pustules and fissured crusts. Previous involvement with this disease, history of contact with sheep or goats, food resources and season all can have a role in epidemiology of the disease.

Key words: Contagious ecthyma, first report, camel, Iran, PCR.

1. Introduction

There is only a little information on camel diseases compared to other species of animals. This may be mainly due to the fact that camel production is usually practiced on a migratory system in remote areas with harsh living conditions that make such studies difficult and expensive to execute [1].

Camels are susceptible to many infectious diseases, some of which have been investigated extensively because they also affect other species of farm animals. Such diseases include trypanosomiasis, anthrax, hemorrhagic septicemia, brucellosis, mange and pox [2]. Pox and pox-like diseases of camels are a group of exanthematous skin conditions, which recently emerge as being of increasing economic importance [3]. They may be caused by three distinct viruses: *Orthopoxvirus cameli* (camel pox), *Parapoxvirus* (camel contagious ecthyma (CCE)) and *Papillomavirus* (camel papilloma virus infection) [3]. The clinical signs of camel pox (caused by *Orthopoxvirus cameli*), CCE and camel papillomatosis are similar and can be confused, especially in the generalized form [4-6] and so far can be distinguished only by virus identification by electron microscopy or polymerase chain reaction (PCR). CCE was first described in Kazakhstan in 1968 [7]. Contagious ecthyma in camels (*Camelus dromedarius*) is a *Parapoxvirus* disease which has

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been described in many countries, such as Mongolia [8, 9], Somalia [4], the former Soviet Union [10], Libya [11], UAE [12], Saudi Arabia [13], Sudan [14] and Kenya [5].

The agent is a DNA poxvirus of the *Parapoxvirus* subgroup, which includes the closely related viruses' pseudocowpox (another cause of orf, like "milker's nodules" in humans), the agent of bovine papular stomatitis (BPS), *Parapoxvirus* of red deer in New Zealand, squirrel *Parapoxvirus* and *Parapoxvirus* of gray seals [15]. There are biological and genetic differences among strains of contagious ecthyma virus, so that cross protection may be limited and vaccines need to target the field strain. The virus is epitheliotropic, usually creating proliferative lesions in the skin of the lips, nostrils, oral mucosa, teats and occasionally the vulva [15].

The disease is characterized by localized lesions, although generalized forms have also been observed, resembling true camel pox [3]. Clinically, nodules appeared on the lips of affected animals, followed in most cases with swelling of the face and sometimes the neck [1]. Papules and vesicles appeared later and within a few days developed into thick scabs and fissured crusts [1]. Lesions occurred sometimes on the face, eyes and nares and in severe cases on the gingival, dental pad and tongue. Death was probably due to starvation caused by the inability of affected animals to graze or to suckle their dams. Healing occurred within 20-30 d in most cases, but sometimes the course of the disease extended up to three months [1]. Secondary bacterial infection or myiasis of affected parts may occur [15]. The possible involvement of insect transmission cannot be excluded. The disease seems to have a seasonal prevalence of an arthropod-borne disease. It appears in early rainy seasons and usually disappears by the end of raining [1]. Orf virus is transmissible to camels, but in comparison with sheep, camel orf infection has received little attention, apart from a limited number of reports describing its clinical and histological features.

It has been assumed that natural infections on pasture are the result of invasion of the virus after skin damage induced by prickly plants or stubble; application of a viral suspension to scarified skin is the established method of inducing orf [16]. Damage to the skin is essential for the establishment of orf infection and the development of typical lesions [16]. The thorny plants damaged the lips, allowing the transmission of *Parapaxvirus* [17]. Definitive diagnosis usually involves identifying the distinctive cross-hatched virus particles in typical early lesions with electron microscopy, PCR, immunohistochemistry or inoculation into known protected or susceptible animals [15]. Vaccination with the material containing CCE virus seemed to be promising. In contrast, camels were not protected after immunization with vaccinia virus and a vaccine against sheep and goat contagious ecthyma [8].

The aim of this study was to submit the first report of contagious ecthyma in Iran and alarm for its economic losses in camel rearing industry. Also in current study, all epidemiological aspect of ecthyma in camel was drown to help vet agent to diagnose this disease easier.

2. Materials and Methods

2.1 Herd and Area

The herd was comprised of 89 camels, including 88 dromedary camels (62 adult females and 26 calves, less than one year old) and one bactrian camel. The male bactrian camel was about three years old. It was bought purposely for breeding and it was why it was grouped with the herd.

The area is in the East of Qom province with about 200 km^2 width, an arid area with hot and dry climate. The temperature in the area fluctuates during the year, and the day had an average temperature higher than 19 °C and an annual rainfall was less than 125 mm.

The dominant vegetation in the region was comprised of the tamarisk and haloxylon trees. Camels had freely grazed in the area. A total of four

herds were included in this study, where three herds were all camels and a herd was a goat within it.

2.2 Examination

Affected animals were carefully examined for clinical signs. During examination, rectal temperature, vital signs, body condition score (BCS), rumination, ruminal movements, dehydration state and state of anemia were recorded, and peripheral lymph node, eyes, lesions in lips and muzzle were examined. Additional data concerning age and sex of animals were recorded.

2.3 Sampling

Biopsies from the lip lesions were collected in sterile containers for virological investigations. Samples were sent to Razi Vaccine and Serum Research Institute for diagnosis. Then, total extracted DNA was used for PCR to amplify a fragment of *Parapoxvirus B2L* gene [18]. The specific oligonucleotide primers used were designed according to Ref. [18].

The herd was monitored to investigate the epidemiological features of the disease.

3. Results and Discussion

3.1 Results

The calves were observed to have suffering from lesions on the lips and muzzle.

In these adult camels, lesions characterized by congested papules and scabs were seen on the lips and nostrils. The lesions were proliferative and were 3 mm to 2 cm in diameter. The animals were sensitive to palpation, thus often resulted in bleeding. In some cases, the mucosa of the mouth and nare were involved, showing haemorrhages in some of the affected parts. The lesions were limited in lips and muzzle, and other parts were not affected (Fig. 1). The animals got emaciated, because they lost their appetite, they were found to be slightly anemic and the ruminal movements as well as rumination decreased. As regards eye examination, conjunctivitis and epiphora were observed. Peripheral lymph nodes were mildly

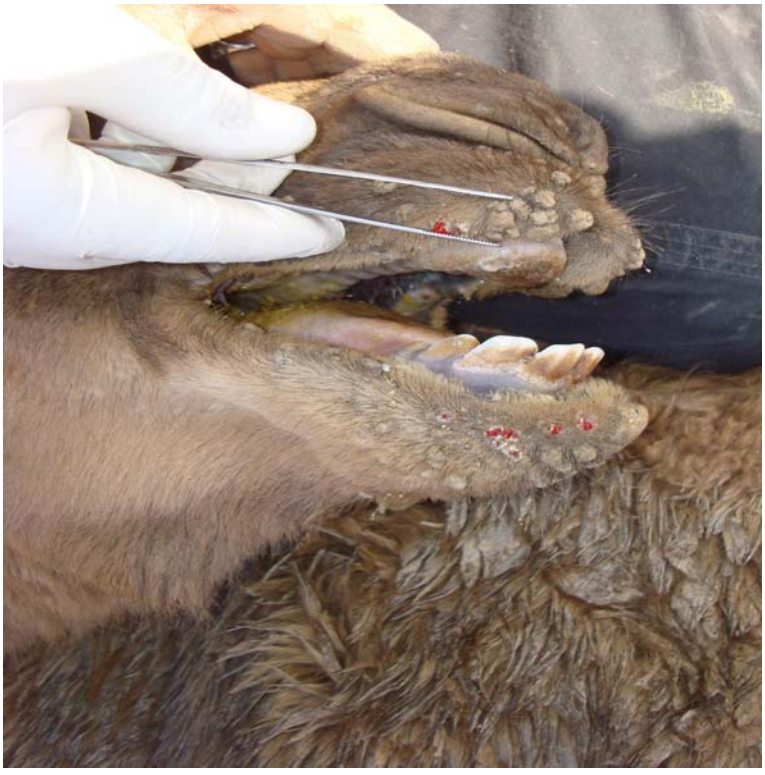

Fig. 1 Proliferative and hemorrhagic lesions with scabs in lips and muzzle in dromedary calves.

enlarged. Affected animals had fever and average rectal temperatures reached 40.5 °C. Furthermore, in some cases, the swelling of the head of the animal was observed. It could be noted that the bactrian camel was significantly affected compared to dromedary camel calves (Fig. 2). The lesions observed in the camels were also seen in the kids, although they received dose of pox vaccine, whereas the adult goat had no observable or clinical signs of lesions.

Congestion and hemorrhage in oral cavity were recorded in eight cases (29.6%), and the swelling of the head was observed in five cases (18.5%).

Diagnostic confirmation was done using PCR. Amplification of the *B2L* gene of camel skin biopsy samples showed a positive result of PCR product with the expected band size of 594 bp (Fig. 3).

Fig. 2 Hemorrhagic popular lesions and fissured crusts in male adult bactrian camel.

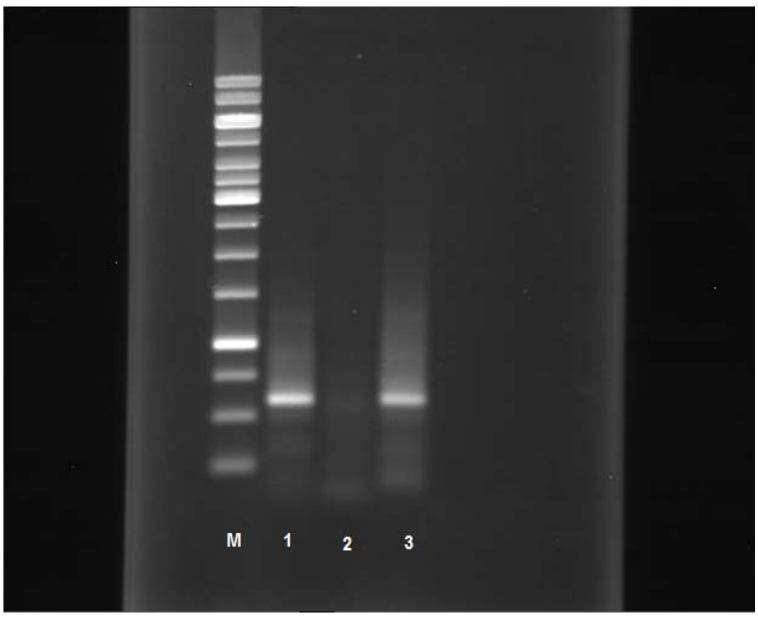

Fig. 3 Identification of CCE virus.

Lane M: 100 bp marker; lane 1: amplification of genomic LT cell DNA infected with contagious ecthyma virus, Kerman/2,000 strain; lane 2: amplification performed on normal skin biopsy; lane 3: amplification performed on skin biopsy with CCE infection.

Table 1 Distribution of the disease based on age, breed, clinical signs and death.

Camels	No.	Numbers of having clinical signs	Numbers of death
Calves (less than one year old)	26	26	1
Adult dromedary female	62	0	0
Adult bactrian male	1	1	0
Total	89	27	1

The prevalence of the disease in the herd, adult camels and camel calves was 30.33%, 1.5%, and 100%, respectively (Table 1). The adult dromedary camels had no clinical signs of the diseases and all were healthy.

The mortality rates in whole herd, adults and calves were 1.12%, 0%, and 3.84%, respectively, and case fatality rates (*r*) in these groups were 3.7%, 0%, and 3.84%, respectively.

The popular lesions progressed to proliferative lesions which were persisted for a week, and then the thick scabs appeared which were difficult to remove. They dried and fell down after 4-6 d, and totally healed in a month time. One animal died due to starvation and secondary infection.

Treatment with antibiotics was administrated only in bactrian camel due to secondary infection and respiratory disorder, which was given for 5 d and was also given a single dose of ketoprofen.

3.2 Discussion

Generally, the limited reports of orf infections in camels are difficult to compare due to the lack of good epidemiological tools as regards to study the infection rates.

The present study is the first report of CCE in Iran [19]. In an earlier study, clinical feature observed was the appearance of pustules on the nose, muzzle and lips and the enlargement of the lymph nodes [20]. Moallin et al. [21] recorded only localized lesions on the muzzle and lips. Similar signs have been reported in Somalia [21] and the Sudan [14]. In another study, generalized lesions involving the distal parts of the legs, the inner thighs and the vagina have also been reported [5]. In this study, also only localized proliferative lesions were founded and other parts were not affected.

In addition, same clinical stages were seen in whole herd, in different reports, it is probably related to the seasonal breeders and therefore many calves are born approximately at the same time.

Morbidity of 100% in calves had been described by other researchers, but this rate was reported to be low (10%-20%) in adult camels [5]. It was also reported that 98% of the cases of orf in camel occur in calves aged less than one year, with a mortality that reached 38% [22]. In another study [8], the morbidity in adults ranged from 10% to 80%, in 2-month-old to 3-month-old suckling camels was between 50%-70% and it reached 100% in 1-year-old animals. The absence of contagious ecthyma in mature animals is probably due to an immune response, which is absent in very young camels; such a pattern has been described for goat kids [23].

In this study, only camel calves less than one year old and one adult bactrian male camel were affected. The adult dromedary camels remained unaffected, as they gained resistance being exposed to the affected animals. The bactrian male camel probably had not been exposed with CCE and therefore had not developed resistance to the disease and was not given vaccination against pox and pox-like viruses. The prevalence and mortality rate is similar to other studies.

Some reports have indicated that outbreaks of contagious ecthyma in camels have occurred in areas where orf is present in sheep [5, 24]. However, in another study in Saudi Arabia [25], camels were not observed to be cross-infected under field conditions, although they were in close proximity with sheep and goats. Gitao [20] reported that the common practice of keeping all camel calves in the same shelter at night

could be responsible for the spread of the virus by contact, and he also proved that the outbreak in camel calves occurred when *Parapoxvirus* infections were also observed in goat kids who were raised in closed proximity place [17, 20]. In the reported causes of the disease in camels, sheep and goats were proven to be carriers and non-carriers of contagious ecthyma. However, in this study, the herd together with the goat got infected.

In most studies, the annual young camel calf is exposed to infection when they start grazing prickly plants. This was the case in Asia [7] and Africa [14, 21, 22]. Factors responsible for this epizootiological feature seem to be the abrasion of the skin of the lips, resulting from that they ate thorny acacia trees at this time of the year when no other source of food was available [1]. The same opinion was also offered by Buchnev et al. [7], who argued that the thorny plants damaged the lips allowing transmission of *Parapoxvirus*.

In this study, tamarisk plant species could have been a contributory factor that transmitted the disease into the herd.

It is demonstrated that disease had a marked seasonality, being associated with the rainy season, and seemed to occur at this particular time every year [1, 7, 21, 26]. This outbreak was observed in early winter (December) and season is also a big factor.

4. Conclusions and Recommendations

This study demonstrated that CCE has a considerable economic impact on camel production in Iran. The effect of this disease was not only due to mortality of camels but also the loss of weight and poor growth rate. Treatment of the infected animals is not easy, as it entails costly antibiotics and drugs. There is a need for an epidemiological study to confirm the presence of the disease. A comprehensive control and prevention program to minimize the occurrence of the disease is very necessary.

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Strategic Path for the Development of Live Pig Healthy Breeding Industry in China

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Abstract: The objective of study in the paper was to analyze advantages, disadvantages, opportunities and threats of development of pig healthy agriculture in China, using systematic analysis and SWOT strategic analysis, as well as to show severe diseases, abnormal fluctuation, misplaced way and versatile environment confronted by pig breeding in China. The paper establishes "double-hug mode", from which pig breeding industry transforms and upgrades to modern breeding industry. Combing with the designed multi-party cooperation mechanism of advantageous regions, urban sale areas and group corporations, it sets up three paths for development of pig healthy agriculture in China, i.e., strategic paths of advantageous regions, urban sale areas and group corporations oriented, with an attempt to provide strategic path reference for transformation and upgrading of pig breeding in China and mode reference for breakthrough of development strategy for pig healthy industry.

Key words: Pig healthy industry, strategic system, path mode, advantageous regions oriented path, urban sale areas oriented path, group corporation oriented path.

1. Introduction

1.1 Background

China is at the historical stage, traditional agriculture is transforming to modern agriculture, land, water and labor resources are insufficient and new urbanization is being pushed rapidly, which all bring severe challenge to development of live pig healthy agriculture. To develop healthy breeding is basic path for development of Chinese live pig breeding. Live pig healthy breeding is a kind of breeding mode with high economic, social and ecological comprehensive efficiency, and development of live pig healthy breeding is a systematic, dynamic and integrated process that realizes innovation relying on strategy, guides by objective standard system, supports with organization system establishment and operates by guarantee system. China's live pig breeding is confronted with many issues, such as severe diseases, abnormal fluctuation, misplaced way and versatile environment, therefore, how to establish strategic system of development for live pig healthy breeding to guarantee consumption, breeding, environment and occupation health and investment and operation of industry chain is critical.

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1.2 References

Pan and Kinsey [1] systematically analyze every link during Sino-US pork production chain procedure and work out that there are scattered farmers, imperfect information and logistics during Chinese live pig industry chain, resulting in low efficiency in operation of live pig industry chain. Williamson [2] delivers that high quality and safe price to terminal of pork industry chain by making use of connected effect of price system for pork industry is good to realize object, which improves overall economic benefits of pork industry chain. Klepper and Simons [3] believes that system innovation is precondition to guarantee mode optimization of live pig industry chain, smooth

transaction and reduce transition cost. Holt and Johnson [4] put forward that we must do well in integration and overall system optimization of live pig industry chain in order to push live pig industry chain to walk towards road with orderly competition, virtuous circle and sustainable and healthy development. Azzam et al. [5] develop a comparative statics model of long-run industry equilibrium in the presence of size-based environmental regulation stringency and applies the model to the US hog industry. Moyer et al. [6] concern about potential, uncertain environmental impacts and an outdated regulatory framework leading to a moratorium on new hog industry operations and a provincial hearing. Meulenberg and Pennings [7] propose a marketing strategic approach to commodity futures exchanges to optimize the (hedging) services offered.

1.3 Significance

The research object of this paper is the strategic path of the development of the pig breeding industry in China. Through the systematic analysis of the environmental conditions of the development of pig breeding industry, the paper points out that China's pig breeding industry is faced with the deep level of the problem, and tries to explore the transition from traditional culture to modern healthy breeding of Chinese pig breeding. It will provide ideas for different types of regional and subject to grasp opportunities and meet challenges, provide the basis for the Chinese government to develop the guiding policy of the pig industry, and provide reference for the research on the path of pig breeding in this field.

2. Analysis of Environmental Conditions

2.1 Development Trend of Live Pig Breeding Industry at Oversea

By comparison development status with trend of live pig breeding industry at oversea, it can be concluded that there is internationalization, intellectualization and welfare trend in live pig breeding industry:

(1) Internationalization: the multi-national live pig slaughtering and processing enterprises begin to do business all over the world and consequently develop live pig breeding bases, slaughtering and processing industry and market circulation. Live pig trading among countries is becoming closer and closer day by day (Tables 1 and 2);

(2) Intellectualization: in the world, monomer live pig breeding scale is expanding continuously, numbers of farms are decreasing year by year (Fig. 1), industrialization level is increasing constantly, so modernization, informationization and intellectualization degree of live pig breeding are improving;

(3) Welfare: the world are paying more and more attention to animal welfare, and new technologies, such as swine breeding system, hybrid vigor use, artificial insemination, all-in-all-out breeding, early isolation and ablactation of piglet and ideal protein theory, are being promoted and applied rapidly. The animal welfare has become an inseparable and important part of food safety sector. In 1974, EU was the first to

Table 1 Importing quantity of live pig in main countries in 2007-2012 (× 10^4 pigs).

Country	2007	2008	2009	2010	2011	2012
USA	1,000.4	934.8	656.5	574.9	571.6	572.5
Russia	37.7	77.0	120.2	72.8	80.0	81.0
Mexico	13.6	8.0	0.7	0.9	1.3	1.5
China	0.3	1.2	0.6	0.6	1.0	1.2
Canada	0.2	0.2	0.3	0.3	0.3	0.2
EU	0.2	0.2	0.3	0.2	0.1	0.1
Global	1,054.2	1,030.5	766.8	659.2	662.1	663.3

Table 2 Exporting quantity of live pig in main countries in 2007-2012 (× 10^4 pigs).

Country	2007	2008	2009	2010	2011	2012
Canada	1,003.2	935.7	637.6	576.1	573.0	574.0
EU	90.1	150.8	221.0	161.4	165.0	170.0
China	0.1	0	0.1	163.6	156.0	160.0
USA	13.7	9.7	2.1	1.5	1.3	1.4
Russia	0.1	0	0.1	0.1	0.1	0.1
Global	1,268.3	1,260.8	1,021.1	902.8	896.4	906.5

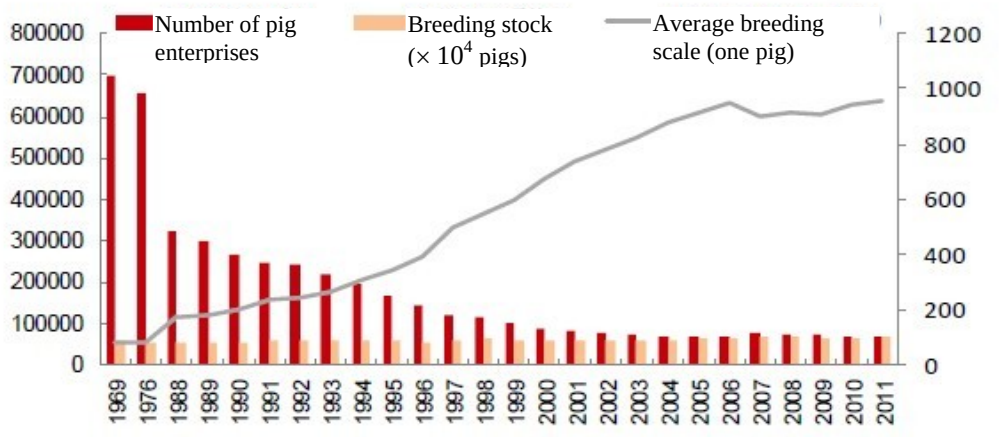

Fig. 1 Statistic of scale level of live pig breeding farm in USA.

establish laws on animal welfare during slaughtering. In January 2006, EU approved specific action plan of animal protection and welfare system perfection, which improved EU animal welfare standard further, integrated relevant animal welfare standards and formed a complete system to be implemented by every country member. From 2007 to 2012, number of animal covered by UK animal welfare system had increased by 50.2%, and cage culture of poultry had been completely eliminated.

2.2 SWOT Analysis of Live Pig Breeding Industry in China

2.2.1 Great and Obvious Influence of the Live Pig Breeding Development in China

Based on analysis of data on the development of pig breeding industry, China is a large country that produces, consumes and exports live pig. At the end of 2012, number of breeding stock was 0.473 billion, among which number of reproductive sows was 49,280,000; numbers of pig to be slaughtered was 0.661 billion, pork output was 50,530,000 tons, accounting for 64.76% of total output of meat and for about 50% of total output and consumption quantity of pork in the word; percent of live pig breeding industry occupied total output value of animal husbandry was up to 47%. Live pig exporting in China plays critically important role in world trading balance.

2.2.2 Five Strategic Issues

The development and expansion of live pig breeding has great influence to increase in farmers' income, food safety, price stabilization and transformation and upgrading, but it is also exposed to development bottlenecks during transformation and upgrading. There are five strategic issues—serious epidemic, abnormal fluctuation, dislocation mode, weak system and environmental risk. It is analyzed through oversea trend that live pig healthy breeding is essential trend for transformation and upgrading of live pig breeding industry. To be specific, based on analysis of data on the development of pig breeding industry and SWOT strategy analysis, the development of live pig healthy breeding in China is faced with the following strength, weakness, opportunity and threat, which are shown in Table 3.

2.3 Establishment of Value SWOT Mode of Development for Live Pig Healthy Breeding in China

Based on healthy life, ecological greening and platform innovation, with reform philosophy and thought, development mode transformation, subject relation optimization as grip, we shall break through challenge and restriction of healthy development of live pig industry chain in China, following trend and fashion of healthy development of live pig industry in the world (Fig. 2).

Table 3 SWOT analysis of development for live pig breeding in China.

	Strength (S)	Weakness (W)
Factors of internal conditions	(1) Scale strength: China is a large country producing and consuming live pig with long history breeding tradition and sound industry foundation. The overall breeding scale has ranked the first few places in the world.	(1) Low matching degree of technology integration: it is difficult for integrating breeding, anti-epidemic, piggery environment and environmental protection techniques, which are necessary for live pig breeding to coordinate and match.
	(2) Capital strength: non-agricultural industry enters into breeding industry, which provides capital condition for developing live pig healthy breeding industry and cultivating new type breeding mode.	(2) Insufficient service system support: the input support, anti-epidemic, equipment support, science and technology, sale service systems, such as variety, feed, vaccine and veterinary drug necessary during development of live pig healthy breeding are incomplete.
	(3) Reorganization strength: live pig breeding is at the volatile transformation stage, leader, follower and displaced persons are participated in competition at different degrees, and the former reorganizes and pushes industrial resource integration to the latter.	(3) Unit breeding scale is very small: according to statistics from Ministry of Agriculture, at present, number of scale pig farms with more than 500 pigs in China has occupied 38.5% of total number of pig farms, however, 60,000,000 individual farmers are still main force, and percent of small-scale scattered breeding is still very high.
	Opportunity (O)	Threat (T)
Factors of external environment	(1) Transformation opportunity: after the 18th Communist Party of China National Congress, China entered into reform and development stage of "five-in-one" and "four modernizations and synchronization", live pig breeding is confronted with the opportunity to production mode transformation and industry level grading.	(1) Threat of serious epidemic: oneness and inadaptability of species, mismatching of anti-epidemic technology and incomplete social service system are important reasons for frequent epidemic occurrence.
	(2) Policy opportunity: central and local government pay attention to development of live pig healthy breeding and issue supportive policies related with breeding, production and processing in succession.	(2) Interruption of abnormal fluctuation: frequent epidemic occurrence, policy control and market influence result in fluctuation of live pig market price, and live pig breeding profit show large fluctuation.
	(3) Market opportunity: the household consumption demands are changing rapidly, and consumption of quality and quantity for live pig and pork production is increasing day by day.	(3) Big risk in environmental change: there are dramatic changes among ecological, epidemic, market, policy and industry environment; water resource is shortage, labor cost increases and land space is limited, which are bad to sustainable and healthy development of live pig breeding.

2.3.1 Use Strength

The strengths of large agricultural and industrialized leading enterprises in the aspect of corporate culture and full industry chain brand, multi innovation and fund allocation, industry chain vertical integration mode and comprehensive innovation platform are fully made use to intensify and improve core competition of healthy breeding of live pig in China.

2.3.2 Internalization Opportunity

We shall grasp the opportunity to support ruminants, such as dairy cow, beef and mutton sheep from the state, break through the species and input research and the development bottleneck of healthy breeding of live pig, meet the demands for high protein healthy and nutritious food from nationals, as well as create vertical and integrated industry chain mode of healthy breeding of live pig.

2.3.3 Deal with Opportunity

We shall explore replacement mode of traditional live pig healthy breeding and search for production way of green and ecological healthy breeding so as to deal with challenges which are brought by high cost, land, labor and water resource and feed raw materials.

2.3.4 Overcome Weakness

During operation of live pig breeding industry chain, we shall overcome weakness of low mismatching of technology integration, insufficient service system support and small scale in unit breeding to reduce objective risk caused by epidemic and abnormal fluctuation of live pig healthy breeding in China.

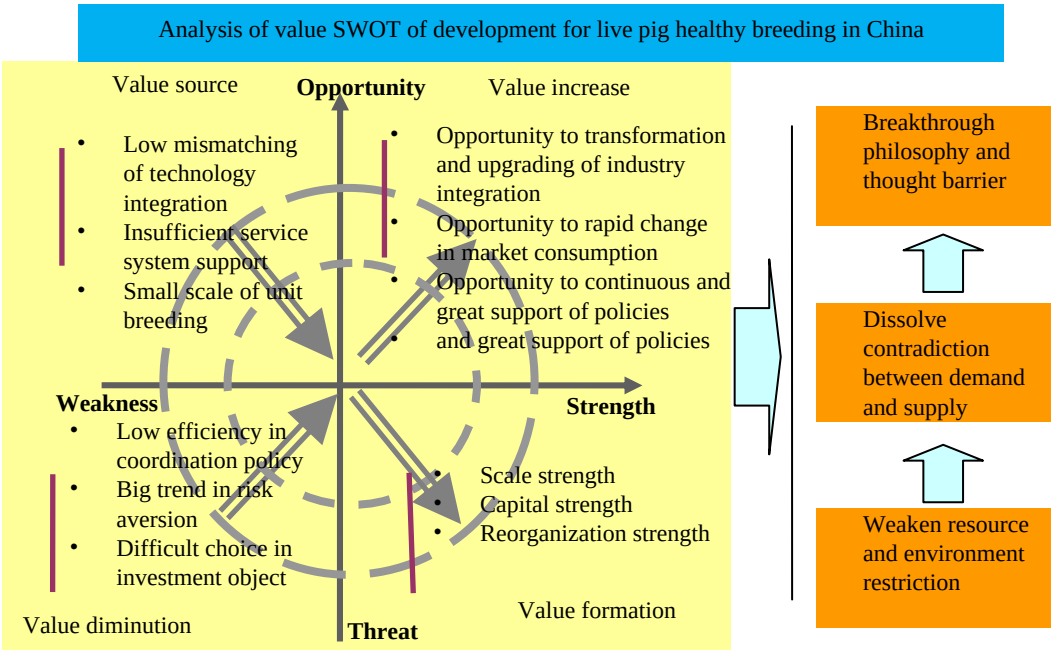

Fig. 2 Value SWOT mode of development for live pig healthy breeding in China.

3. Strategic Thought

It is necessary for healthy transformation, upgrading and optimization of live pig industry chain to intensify natual, symbiosis and implantation energy, improve breeding development level and promote structure evolution of live pig industry chain. We shall establish value/energy/level formation and enlargement mode that guides live pig healthy breeding from two dimensions of development level and evolution stage to provide system mode and reorganization foundation for resource, formation, transformation, enlargement, recycling and spinal upgrading for value/energy/level for live pig healthy breeding as well as to provide power source for formation and influence of thought and conscienceness, logistic and fund, energy and value flows for live pig breeding. In Fig. 3, there are three spinal modes [8], which are also called “double-hug mode”. Three spinal modes include value, energy and evolution spinals.

3.1 Value Spiral

Value spiral is also called as “double-hug model” of live pig breeding transformation, upgrading and optimization. There is potential difference between live pig healthy breeding and traditional live pig breeding, and work is done through potential energy to affect transformation of live pig industry chain. Interaction between live pig healthy breeding and tranditional live pig breeding will produce game competition symbosis through field energy to affect upgrading of live pig industry chain. Live pig healthy breeding is ultimate development diretion to form new energy and produce spinal and hierarchical cycle energy under influence of subject, mechanism and platform to affect optimization of live pig healthy breeding value chain. Value dynamic evolution of live pig healthy breeding development is affected by energy during the process.

3.2 Energy Spiral

Energy spinal refers to iterative spiral among narual, implantation and symbiosis energies. Power source of live pig healthy breeding development mainly replies on narual, implantation and symbiosis energies and shows potential, field and power energy. Natural energy mainly means energy accumulated, inherited

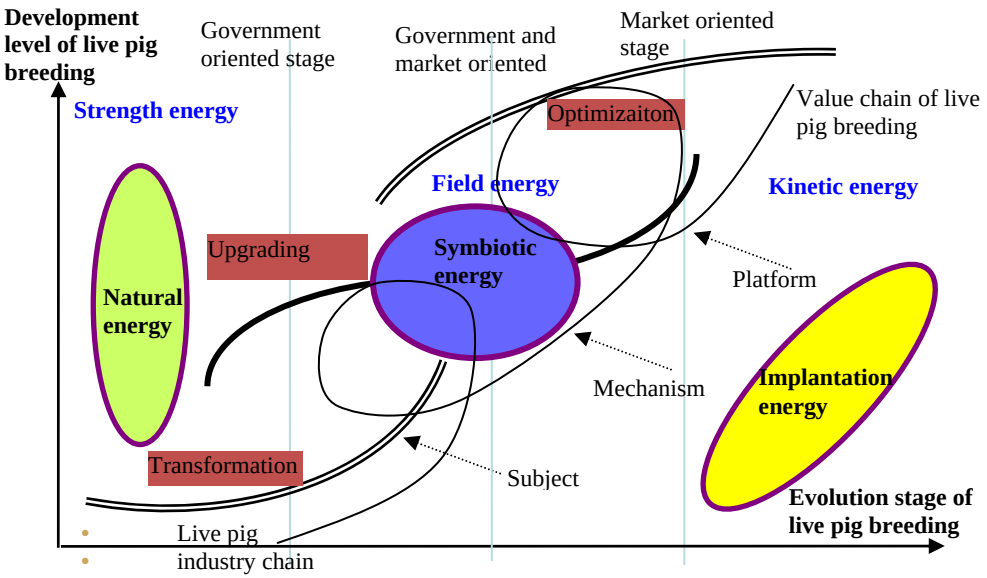

Fig. 3 Three spiral modes of value and energy flow influence of development for live pig healthy breeding.

and left by traditional live pig breeding, symbiosis energery means energy cycle from competition and cooperation symbiosis among relevant subjects of live pig breeding industry chain, and implantation energy means energy injected through external innovation platform or negentropy. Natural energy accumulates to some degree to form potetial energy, symbiosis energery interacts to some degree to form field energy, and implantation energy is impacted externally to some degree to form power energy. Potential, field and power engeries take effect to live pig healthy breeding industry chain together to push transformation, upgrading and optimization of live pig breeding both internally and externally, which promotes the accelerated differentiation and survival of the fittest of subject organization for live pig breeding.

3.3 Evolution Spiral

Evolution spinal refers to evolution of development level for live pig healthy breeding from lower, medium to senior level. It mainly means the spiral line on the right, under the influnce from government, consumption, processing and operation and service subjects. The development of live pig healthy breeding will go through evolution from government-oritented stage to both government and market oritented stage and then to market oriented stage, and show trend of evolution from lower, medium to senior level, as thought and conscieous streams, material and fund streams and energy and value streams exchange constantly.

4. Strategic Path

The path mode of Chinese live pig healthy breeding refers to set of subjects, such as enterprises, cooperatives, farmers, associates and joints, under influence from both market and government, and are composed of development strategy, action path and mode of breeding market system, complete government function and market and government linkage in order to promote establishment and operation of large breeding bases and industry chain joint organization. To realize five healthy targets of live pig healthy breeding, push the establishment and operation of live pig breeding industry chain organizations, and perfect the profit drive and the promotion, expansion and co-augmentation of mechanisms of live pig breeding, depending on orientation subject, target standard, organization type

and mechanism guarantee differences of live pig healthy breeding, the path mode of live pig healthy breeding development can be divided into development path modes of advantageous regions, urban sale areas and group corporations oriented.

The study mechanism framework of strategic path for live pig healthy breeding development is a system analysis framework, which is composed of three dimensions of advantageous regions, urban sale areas and group corporations oriented [9]. Each mechanism corresponds and correlates with target, organization and mechanism systems, providing framework foundation for system design of strategic path for live pig healthy breeding development **(**Fig. 4).

4.1 Strategic Path of Advantageous Regions Oriented

It is necessary for live pig healthy breeding development to form and optimize path mode of live pig healthy breeding of advantageous regions oriented.

4.1.1 Basic Composition

With advantageous region oriented, it is path mode that perfects and promotes market and government function systems of live pig healthy breeding; with advantageous region oriented, it is path mode that breeds and establishes live pig healthy breeding bases and industry chain (supply chain) alliance; with advantageous region oriented, it is path mode that breeds and establishes market and government function systems, live pig healthy breeding industry bases and industry chain (supply chain) alliance joint mechanism, which is shown in Fig. 5.

4.1.2 The Formation and Influence

The formation and influence of path mode for live pig breeding development of advantageous regions oriented is consequently cumulative. Depending on rich resource, advantageous regions and traditional industry, it forms live pig healthy industry of advantageous regions oriented and undertakes advanced breeding mode, technologies, philosophies and systems of market oriented, which provides very sufficient and stable market supply, and effective production base and sale breeding mode for live pig healthy breeding development.

4.1.3 The Influence

The formation and optimization of path mode of live pig healthy breeding development of advantageous regions oriented has great influence. The complete government system is external factor for formation and optimization of path mode for live pig healthy breeding of advantageous regions oriented, while live pig healthy breeding industry base with scale, intensification and organization is internal factor for formation and optimization of path mode for live pig healthy breeding of advantageous regions oriented.

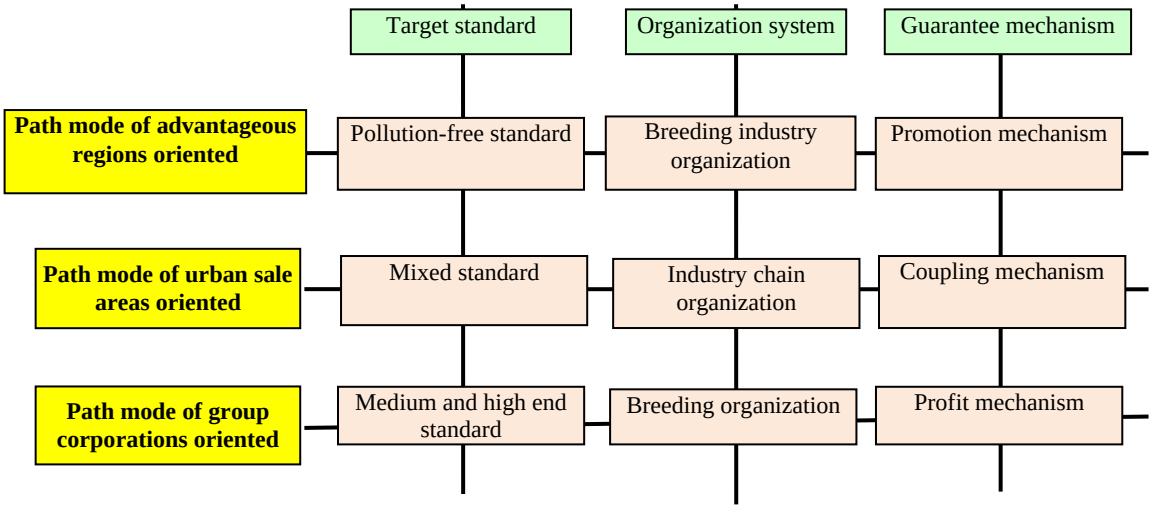

Fig. 4 Mechanism framework of strategic path for live pig healthy breeding development.

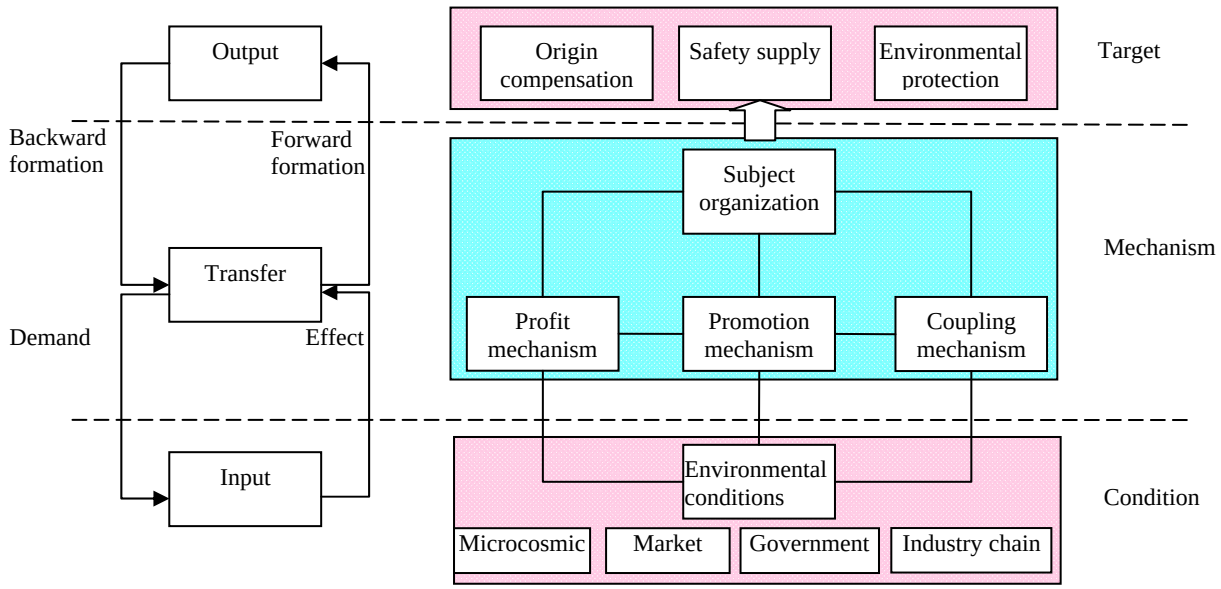

Fig. 5 Study framework of strategic path of live pig healthy breeding development of advantageous region oriented.

4.1.4 Complementary Relationship

With advantageous region oriented, to perfect and promote market and government function systems of live pig healthy breeding is external environment; with advantageous region oriented, to breed and establish path mode of live pig breeding healthy breeding bases and industry chain (supply chain) alliances is internal support; with advantageous region oriented, to breed and establish path mode of market and government function systems, live pig healthy breeding industry chain bases and industry chain (supply chain) alliance joint mechanism is development trend.

4.2 Strategic Path of Urban Sale Region Oriented

It is necessary for live pig healthy breeding development to form and optimize path mode of live pig healthy breeding of urban sale regions oriented.

4.2.1 Basic Composition

With urban sale region oriented, it is path mode that perfects and promotes market function and government function systems of live pig healthy breeding; with urban sale region oriented, it is path mode that breeds and establishes live pig healthy breeding bases and industry chain (supply chain) alliance; with urban sale region oriented, it is path mode that breeds and establishes market and government function systems, live pig healthy breeding industry bases and industry chain (supply chain) alliance joint mechanism, which is shown in Fig. 6.

4.2.2 The Formation and Influence

The formation and influence of path mode for live pig breeding development of city urban regions oriented is backward introductory. Depending on rich support and guarantee systems (tax preference, bank and government cooperation, financial support) of consumption market and government in large cities, with support for large enterprise and bases in urban sale regions to establish live pig healthy breeding bases as main form, it provides support in order to satisfy safety consumption demands for live pig and pork, and provides guarantee conditions in order to implement “vegetable basket project” and food safety action plans in urban sale regions, as well as provides mode model for sound interaction relationship among “quality-quantity-price” in large sale regions.

4.2.3 The Formation and Optimization

The perfect and complete market function system is external factor for formation and optimization of path mode of live pig healthy breeding development of urban sale oriented; the reasonable profit increase, the

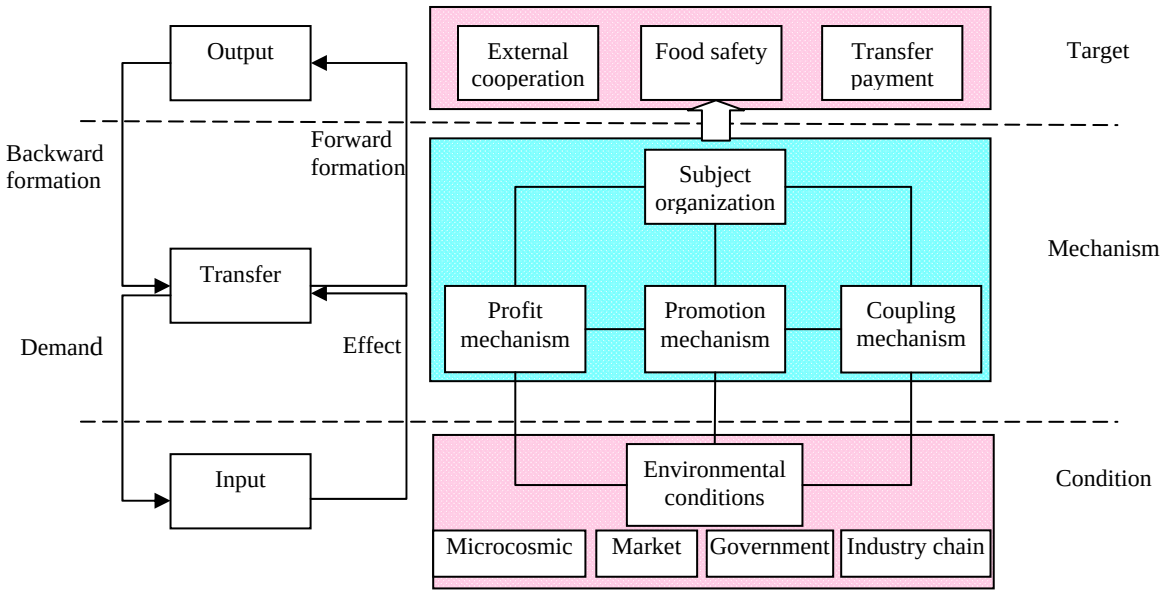

Fig. 6 Study framework of strategic path of live pig healthy breeding development of urban sale region oriented.

adjustment and allocation mechanisms among origin and urban regions, as well as the suitable promotion and cooperation mechanism are internal factor for formation and optimization of path mode of live pig healthy breeding development of urban sale oriented.

4.2.4 Complementary Relationship

With urban sale region oriented, to perfect and promote market and government function systems of live pig healthy breeding is basic power; with cooperation between origin and urban regions oriented, to breed and establish path mode of live pig breeding healthy breeding bases and industry chain (supply chain) alliances is replacement measure; with origin and urban regions oriented, to breed and establish path mode of market and government function systems, live pig healthy breeding industry chain bases and industry chain (supply chain) alliance joint mechanism is development guarantee.

4.3 Strategic Path of Group Corporations Oriented

It is necessary for live pig healthy breeding development to form and optimize path mode of live pig healthy breeding of group corporations oriented.

4.3.1 Basic Composition

With group corporations oriented, it is path mode that perfects and promotes market function and government function systems of live pig healthy breeding; with group corporations oriented, it is path mode that breeds and establishes live pig healthy breeding bases and industry chain (supply chain) alliance; with group corporations oriented, it is path mode that breeds and establishes market and government function systems, live pig healthy breeding industry bases and industry chain (supply chain) alliance joint mechanism, which is shown in Fig. 7.

4.3.2 The Formation and Influence

The formation and influence of path mode of live pig breeding development of group corporations oriented is interactive. Depending on close and interactive full industry chain (supply chain), alliance of live pig healthy breeding industry chain (supply chain) and live pig healthy breeding service management company, with increasing enterprises' market operation ability as purpose, by way of standard improvement, customer segment and channel establishment, it has influence to drive and lead full industry to path mode of live pig healthy breeding development.

4.3.3 The Formation and Influence

The formation and influence of path mode of live pig breeding development of group corporations oriented

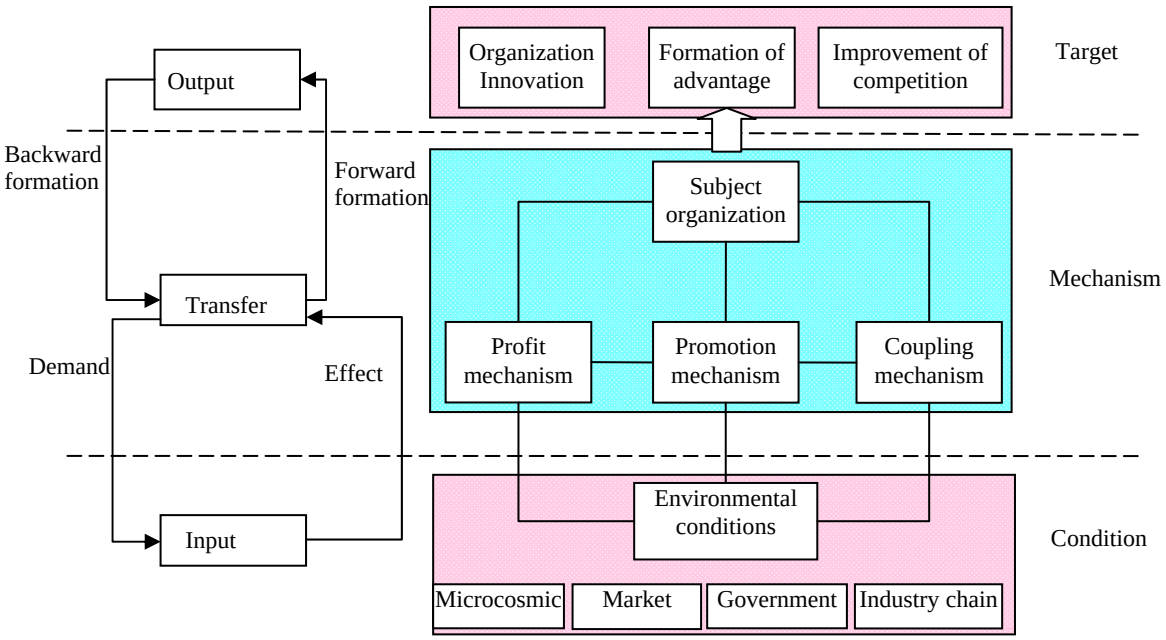

Fig. 7 Study framework of strategic path of live pig healthy breeding development of group corporations oriented.

is affected greatly by target standard system. The target standard system, market function (brand) system and "quaternity" joint mechanism of live pig healthy breeding is external factor for formation and optimization of live pig breeding development of group corporations oriented; internal pursuit of corporation development and leadership-system-culture is internal factor for formation and optimization of live pig breeding development of group corporations oriented.

4.3.4 Complementary Relationship

With group corporations oriented, to perfect and promote path mode of market and government systems of live pig breeding development is basic condition; with group corporations oriented, to breed and establish path mode of live pig healthy breeding industry base and chain (supply chain) alliance is support guarantee; with group corporations oriented, to breed and establish path mode of market and government function systems and live pig healthy breeding bases and industry chain (supply chain) alliance joint mechanism is ultimate purpose.

5. Conclusions

To develop healthy breeding is basic path for development of Chinese live pig breeding. Live pig breeding in China is confronted with many issues, such as severe diseases, abnormal fluctuation, misplaced way and versatile environment. The development of live pig healthy breeding is a systematic, dynamic and amalgamation process that realizes innovation relying on strategic breakthrough, guides by objective standard system, supports with organization system establishment and operates by guarantee system, while transformation and upgrading of Chinese live pig breeding need to explore various development paths and modes of live pig healthy breeding of advantageous regions, urban sale areas and group corporations oriented. It is worthy to note that path mode of live pig healthy breeding development is affected by demand environment, target condition and subject organization. The comprehensive influence among these environmental conditions will have influence to change in subject action of live pig breeding industry chain.

Acknowledgments

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The Main Causes of Calf Mortality in Dairy Farms in Poland

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Abstract: Calf mortality is one of important problems of calf rearing in dairy farms worldwide. Besides, several noninfectious factors, such as management around birth, colostrum management, calf housing, feeding system, hygiene and pathogens, play an important role in calf rearing. The aim of the study was to show the most common causes of mortality of calves up to 90 d of their lives. Some data are available concering calf rearing management on small and medium size dairy farm typical for Polish regions. The research was conducted in seven selected herds of Polish Holstein-Friesian cows located in South of Poland. Data on calves mortality covered the period of three years from 2004 to 2007 and were collected using medical documentation and medical inquire in the farms. All evidence was enrolled untill three months of age of calves. There were 1,800 calves tested. The influence of such factors as maintaining system (free stalls barn and stalls barns), feeding systems and herd size on falls of calves was examined. Overall, mortality throughout the three months of study period was diarrhea, which increased the risk of death among calves younger than 90 d of age. Also, respiratory system disorders were the common cause of loss of calves. The calf mortality rate during 90 d in all herds registered in free stall barns was 61% and in stalls barns was only 29%. Effect of pneumonia in free stall barns was 18% and in stall barns was 29%. In all groups, calf mortality rates increased with increasing herd size.

Key words: Dairy cattle, calf health, calf mortality.

1. Introduction

Healthy calves form the basis of any successful cattle production system, from both economic and animal welfare points. The most common health problem is diarrhea in newborn calves, which causes great economic loss to dairy producers [1]. Diarrhea is a complicated, multifactoral disease with noninfectious factors and numerous infections. In breeding of dairy cattle very important is proper rearing of calves. Healthy, well-reared calf ensures the breeding progress in milk production. Also, movement and condition of cows are the factors that support calving. Calves which are intended for replacement in the herd should be characterized by good health.

The focus on disease prevention rather than veterinary treatment means that an efficient health supervisory system is necessary.

Assessment of animal welfare requires development of new strategy based on information obtained by many measurements. Welfare measurements may differ in accuracy, relevance and relative importance for overall well-being. Because of an increasing interest in assessment of calf welfare, it is necessary to collect data that can supply information about the quality and animal-friendly way of animal housing and care [2]. To determine the influence of different systems of keeping healthy calves on welfare, it is necessary to prepare one system with welfare standards that could be used under various conditions. The aim of the study was to evaluate and compare welfare of calves on selected dairy farms. Proper care of the calves during feeding of colostrum could protect animals from health problems later in life. Unfortunately, despite of the best intentions and well done work, calves are not always protected against

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diseases [3, 4]. Most common health problems of calves could be divided into a couple of groups depending on easily calving. The first group contains cases of stillborn calves with weak calf syndrome and birth defects, the second group contains cases of diarrhea and the last group contains the latest occurring respiratory diseases. However, many of these factors are not modifiable on commercial dairy farms.

What should also be mentioned is the possibility of the birth of weak or deformed calves by the administration of certain drugs during pregnancy of the cows [5]. Most often this occurs because of lack of knowledge and uncontrolled usage of drugs without consultation or supervision of a veterinarian, who should be the most competent person in such cases. Some drugs result in developmental defects or fetal death. The most common birth defects in of calves include lung atelectasis, changes in the brain, no anus and same others. Also, what should be important to remember is the genetic base of these changes that may occur through inattentive matching bulls for artificial insemination or coverage of closely related animals [6].

2. Materials and Methods

The study was designed as a case-control study and the dependent variable in all statistical models was the type of farm.

Data consisted of information gathered over three years (2004-2007) period in a production environment. Data on falls of calves were analyzed on the basis of medical documentation and medical history in individual farms on the material counting a total of 1,800 pieces of cows Polish black and white Holstein-Friesian. Cows involved in tests were used in seven farms located in the provinces of South Poland.

To achieve good similarity in structure, the type of farm, the type of cow barn (free barn stall or stalls barns) and the number of dairy cows were used as further criteria. Suitable farms were available in the same geographical region. A questionnaire was used to collect information during a face-to-face interview with the farm owner or manager. Space of interest were farm characteristics, health status of animals, calf housing and feeding, focus on calves within the 90 days of life, management practices around calving and birth, as well as hygienic measures. The percentage of contaminated places was documented (0 to 100%).

Respiratory tract disease was defined as several increased respiratory sounds at lung auscultation or as moderately increased respiratory sounds together with additional signs, such as dyspnea, coughing or nasal discharge.

On all farms, calves were usually separated within 24 h after birth. On almost all farms, the attendants or manager stated that each calf received colostrum within 6 h after birth or some were allowed to suckle their dam.

On the bigger farms, calves were housed individually after birth, usually for 1-12 weeks, with a median of six weeks. Calves were grouped after weaning. All calves were housed on long straw during pre-weaning period. Individual housing was cleaned daily or farmer indicated that fresh straw was added if necessary, and pens were cleaned after the calf left the box.

On most of the farms, milk or milk replacer was fed restricted to 10% to 12% of the calf body weight (BW), usually in two meals per day. On none of the farms was found the amount of solid food eaten by the calf before weaning. Hay was offered to the calves from the second week of the life. On the bigger size farms, calves had free access to concentrates, starting within the first three weeks of life.

The calves were categorized as clean or mildly dirty in all farms. The calving area was cleaned after each calving in every farm.

3. Characteristics of Evaluated Farms

3.1 The Family Farm (A)—Polish Black and White Holstein-Friesian Cattle

The free barn stall maintenance system with deep

litter concerns 55 dairy cattle with an average yield of 7,500 kg of milk per lactation. Milking takes place in the parlor of "herringbone stall". There is no grouping nutritional feed station. Age of first calving on farm A is ≥ 24 months. The length period is between 275 d and 302 d.

3.2 The Family Farm (B)—Polish Black and White Holstein-Friesian Cattle

Positions of system maintenance were long straw and manual feed. There are 24 cattle with an average yield 5,500 kg of milk per lactation. Milking is done using pipeline milking machine.

3.3 State Farm (C)—Polish Black and White Holstein-Friesian Cattle

Positions of system maintenance were long straw and manual feed inflicted by hand. There are 40 cattle with an average yield 6,500 kg of milk per lactation. Milking is done using pipeline milking machine. Calving at ≥ 22 months was practice on farm B. The length period was between 277 d and 298 d.

3.4 The Family Farm (D)—Polish Black and White Holstein-Friesian Cattle Varieties of 75% Share of Holstein-Friesian Genes Race

The maintenance system in the free barn was deep bedding, while the nutrition system was a mixed feeding. There were 60 cattle with an average yield 6,500 kg of milk per yield. Milking takes place in the parlor of "herringbone stall". Age at first calving is between 23 months and 24 months. The length period is between 270 d and 295 d.

3.5 The Family Farm (E)—Polish Black and White Holstein-Friesian Cattle

The free barn stall maintenance system was deep bedding, while the nutrition system was a mixed feeding. There were 259 cattle with an average yield of 6,500 kg milk per lactation. Milking takes place in the parlor of "herringbone stall". Calving is at age ≥ 23 months. The length period is < 272 d and > 273 d.

3.6 The Family Farm (F)—Polish Black and White Holstein-Friesian Cattle Varieties of 75% Share of Holstein-Friesian Genes Race

The free barn stall maintenance system was deep litter bedding system, while the nutrition system was a mixed feeding. The number of 250 cattle with an average yield 7,500 kg of milk per lactation. Milking takes place in the parlor of "herringbone stall". Calving period is between 22 months and 24 months. The length period is ≥ 280 d.

3.7 The Family Farm (G)—Polish Black and White Holstein-Friesian Cattle Varieties of 75% Share of Holstein-Friesian Genes Race

The free barn stall maintenance system was deep litter bedding system, while the nutrition system was a mixed feeding. The number of 104 cattle with an average yield 8,500 kg of milk per lactation. Milking takes place in the parlor of "herringbone stall". First calving is at age ≥ 22 months. The length period is ≥ 278 d.

The work is based on interviews carried out with environmental survey and based on the documentation breeding, i.e., card heifer-cow farms located in South of Poland. Intention of the interview was to establish the most common causes of calves' mortality up to 90 d of age.

4. Results

The results concerning the impact of such various factors as how to maintain free barn system or tie-stall system, farm size, breed and causes of falls of calves are shown graphically in Figs. 1 and 2 and Tables 1-5. Fig. 1 presents the result of calving during year of 2004 depending on maintenance system, like tie-stall and free barn system. The year has been divided into seasons.

The collected data show that in farms with free barns system, distribution of calving during the year

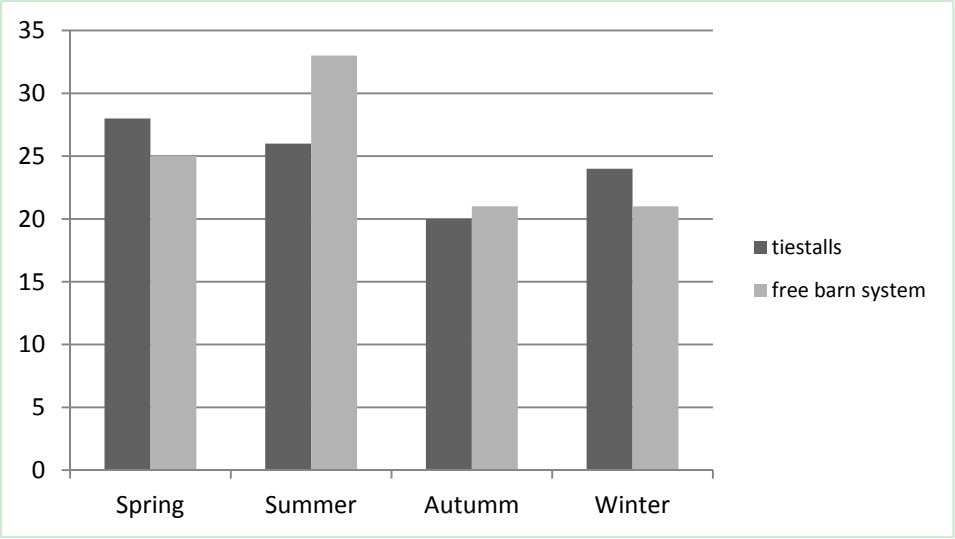

Fig. 1 The distribution of calving in the year of 2004 depending on system maintenance.

Table 1 Causes of calves mortality in free barn housing system.

Free barns housing	Percentage
Diarrhea	61%
Postnatal respiratory disorders	18%
Perinatal	14%
Malformations	3%

Table 2 Causes of falls of calves in tie-stall system.

Barns housing	Percentage
Diarrhea	29%
Postnatal respiratory disorders	29%
Perinatal	28%
Malformations	14%

Table 3 Causes of calves mortality on the farm A (50-100 pieces).

Barns housing	Percentage
Diarrhea	46%
Postnatal respiratory disorders	38%
Perinatal	8%
Malformations	4%
Others	4%

Table 4 Causes of calves mortality on farm B (101-200 pieces).

Barns housing	Percentage
Diarrhea	33%
Perinatal	20%
Postnatal respiratory disorders	27%
Malformations	20%

was the highest number in the period of calving years (33%), while in the holdings of the tie-stall system, calving season is distributed relatively equally throughout the years (Fig. 1).

Table 5 Causes of calves mortality on the farm C (201-300 pieces).

Barns housing	Percentage
Diarrhea	69%
Perinatal	10%
Postnatal respiratory disorders	8%
Malformations	8%
Others	5%

Based on the interpretation of the results, it was found that in the tie-stall maintain system, calves mortality are caused by diarrhea and postnatal respiratory disorder (29%) and no proper proceedings of the calf after delivery, which is at the level 28% (Table 2).

The impact of the number of cows on the distribution of calving on the farm A was the highest in the summer months (44%) and the lowest in the fall and winter range (14%-16%).

On farms B and C, the difference between the distribution of was slightly more visible in farm C. This difference is 10% in favor of farm C. These differences are shown in Fig. 2.

Considering size of farms, number of cows and calves in a farm, the cause of mortality of calves in these farms was diarrhea (farm A: 46%; farm B: 33%; farm C: 69%).

The data given in Tables 3-5 show that the causes of mortality in these farms are as follows: postnatal respiratory disorders in farm A are 38%, in farm B are

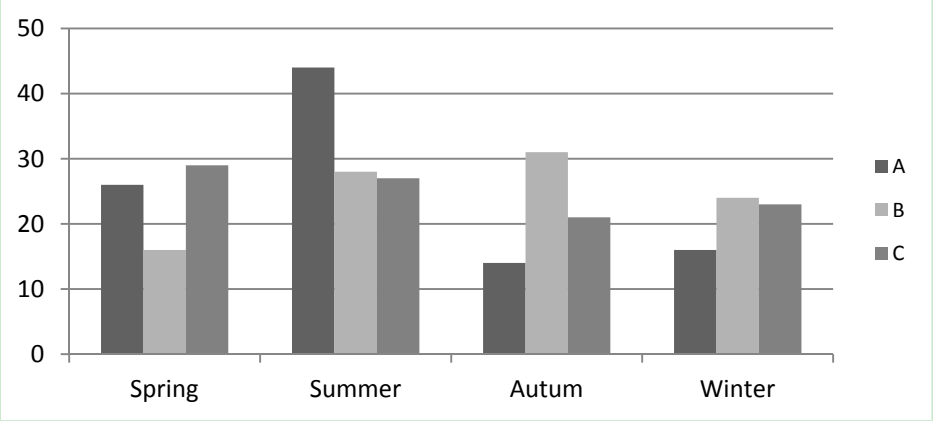

Fig. 2 The distribution of calving in 2004 depending on the size of farms (%).
Classification of households depended on the number of cows. A: 50-100 pieces; B: 101-200 pieces; C: 201-300 pieces.

27% and in farm C are 8%; the proceedings of calf after birth in farm A are 8%, in farm B are 20% and in farm C are 10%, Malformation cases on farm A are noted 4%, in farm B are 20% and in farm C are 8%.

In addition, in the farm A and C, some live-born calves with a genetic defect called molformations were recorded, which causes death soon after birth. Percentage of mortality in both farms was 5% (Tables 3 and 5).

In the farm C, the largest mortality of cows calving was recorded to be caused by diarrhea (Table 5).

5. Discussion

This study surveyed and described management practices in calf rearing on dairy farms in Polish free land to identify risk factors of calf's mortality on those farms. Except for farm characteristics, the type of the cow facilities, breed and caretaker were similar among farms. In this study, the presence of diarrhea was significantly associated with herd size. Similar results concerning the association between the appearance of diarrhea and herd size were obtained in Ref. [7]. Another factor that might influence the risk for diarrhea is the time which the calf spends with a mother in the calving area. Especially, poor hygiene condition was not a risk factor on the examined farms. The importance of an adequate colostrum supply and therefore the passive transfer of immunoglobulins for calf health is well known. In the first week of age, calves are commonly advised to be housed individually, because it may lead to a decreased pathogen load [8]. Moreover, grouping relatively late in the calves' life (median of six weeks with interquartile range 4-8 weeks) could have positively influenced morbidity, because the risk for diarrhea is the highest in the first three weeks of their life [9]. On all farms which have been visited, calfs were fed with whole milk. Additional advantages of feeding whole milk are easier and less error-prone than feeding milk replacer. The farms had similarity feeding management system. Based on the research, it can be concluded that the most common cause of mortality of live-born calves was diarrhea. Similar conclusions were reached in Refs. [3, 4, 10], in which it was also reported that because of diarrhea, especially in the first period of life of calves, the mortality was observed. This is also confirmed in Ref. [5] that the steady intensification of livestock farming leads to a high concentration of animals in a small area. This phenomenon favors the spread of infectious diseases, particularly in calves. Currently, 80% of cases of disease in this age group are disorders of a diarrhea and respiratory syndrome, which was also demonstrated in this work. Similar values were received in Ref. [11], which confirmed that diarrhea and respiratory diseases accounted for approximately 80% of cases of disease in calves and were the main

cause of business failure and economic losses. In this study, the presence of respiratory tract disease was significantly associated with diarrhea. Furthermore, the number of farms with calves suffering from respiratory tract disease was lower.

Stefaniak [12] argued that the period of neonatal diarrhea was one of the main reasons of morbidity and mortality of calves. It is estimated that the period of acute diarrhea causing 75% calf deaths was three weeks. Especially, dangerous for calves is infectious diarrhea. They are most commonly caused by enterotoxigenic strains of *Escherichia coli* and *Rotavirus*, with which particularly dangerous infections are mixed. If the calf is affected with diarrhea, it significantly increases the risk of other diseases, especially respiratory organ infection.

Sobiech [11] has distinguished three periods of the incidence of diarrhea: postnatal (1-4 d), weaned and the transition to a newborn calf period. Normally, illness lasted about a week, and the recovery period was 3-5 d. The reasons are the following: dietetic, bacterial, fungal, viral, toxic, parasitic, allergic and stressful.

A major health problem in the case of diarrhea of calves as well as in other animals is the dehydration caused by increased loss of fluids in the body and reduction of their delivery with milk. Long-term and persistent diarrhea can lead to disorders of homeostasis with fatal outcome [13].

According to some opinions of Stefaniak [12], quite important but often underestimated, the cause of the diarrhea may be the shortage of calcium in milk, which extends time of clotting of casein in the abomasum and threatens emergence of a "full stomach diarrhea". Regardless of the cause, primary intestinal dysfunction is conducive to connect with the infectious agents, risk of diarrhea is directly proportional to the concentration of pathogens in the environment, which depends on how are the wagon, cleaning and disinfecting stalls, air quality, walls and other environmental factors.

According to Refs. [14, 15], respiratory diseases affect over 90% of the calves, the percentage of dead relative to their total number is 5.3%, and the percentage of deficiency reaches 4.5%. Respiratory diseases of calves, known as "the flu calves" or enzootic bronchopneumonia (EBPC), are the main economic problem in rearing calves in both herds of cattle milk and meat production profile.

The cause of influenza calves is complex collaboration of environmental factors and microorganisms. Among the factors, stress factors are non-communicable, but they play an important role, in such as a transport, a dietary mistakes, a change of environment, a method of animal care, a microclimate of premises and veterinary treatments [14].

The problem with bovine viral diarrhea (BVD) in a herd usually occurs as a result of purchase of new animals that are infected temporarily or permanently. Chance of being infected can result from contact with animals on pasture in the neighborhood or wild ruminants and during maintenance [2].

The main problem in controlling BHV-1 infection is the presence of the virus after infection in ganglia and peripheral nerve fibers throughout life of the animal host. Re-virus excretion may take place almost always after activation of stress factors (e.g., parturition). Other illnesses and the use of glucocorticoids also run shoemaking. Therefore, each animal is seropositive to the sower of the potential of the virus [2].

Proper breeding developments affect not only the growth of animals and their health, but also for the development of organs, which in productive age can decide later use value of youth destined for the renewal of the dairy herd, as well as cost rearing and use of milk. Especially, the important is the period of feeding the liquid with feed when calves are not yet fully developed rumen and the enzymatic activity the digestive tract is limited. The improper nutrition and keeping calves during this period make respiratory and digestive tract worse, consequently leading to a

reduction in their subsequent productivity of milk [16].

Rearing of healthy and well-developed calves intended to reproduce the dairy herd depends on efficiency of cattle breeding and use [17]. Mistakes in nutrition, lack of maintenance and increased concentration of calves lead to increased stress and the lower immunity. The results of incorrect rearing are substantial financial losses due to mortality of animals and the weak and sick heifers, which in the future will be difficult to calve and will produce small amounts of milk of low quality [6].

Stefaniak [12] and Sobiech [13] found that we can prevent the occurrence of diseases neonatal period by proper handling of the calf after birth, caring for quick and correct colostrums-drinking calves, taking care of the hygiene of the environment in which calves are born and reside, disinfection of premises and mulch as well as specific active immunization of pregnant cows.

6. Conclusions

Many calf rearing management factors were similar between the visited farms, while variables significantly related to diarrhea on the farm were farm size.

The most common cause of calve mortality before reaching 90 d of age is diarrhea. The second most common cause of calve losses is respiratory—postnatal respiratory disorders and digestive system—catarrhal enteritis. It is concluded that different housing system in dairy farming can be a problem during 1-90 d on coexisting issues concerning calf management and health. When dealing with calf mortality problems, it can be helpful to bear in mind of these coexisting issues and include analyses of the entire herd situation.

Bovine perinatal mortality is increasingly being viewed as a dairy cattle welfare problem.

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Mechanism Analyses for Elucidating the Role of LOXL2 Silencing in Hepatocellular Carcinoma

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Abstract: The paper aimed to study the effect of lysyl oxidase-like 2 (LOXL2) on hepatocellular carcinoma (HCC) and explore the biological mechanisms of tumorigenicity and progression in HCC. The authors used four HCC cell lines to identify LOXL2. A lentiviral vector containing LOXL2-siRNA was constructed to silence the LOXL2 gene in SMMC-7721 cell line, and mRNA of the target gene was detected by real-time polymerase chain reaction (RT-PCR). The effect of LOXL2 silencing on the growth of SMMC-7721 cells was explored with flow cytometry profiling and BrdU labeling. Downstream genes of LOXL2 were selected by microarray and verified by Western Blotting. In the results, LOXL2 expression was significantly up-regulated in four types of HCC cell lines, therefore, SMMC-7721 cell line was selected for further exploration. When SMMC-7721 cell line was infected with LOXL2-siRNA, the expression of LOXL2 mRNA decreased. The silencing of LOXL2 resulted in the cell cycle arrest at the G1-phase, the increased apoptosis and the decreased growth of SMMC-7721 cells on the indicated days by BrdU. Moreover, the MDM2, BIRC3, CDC42, FOS and TGFBR2 genes were selected and verified to be the downstream genes of LOXL2. In conclusion, LOXL2 contributes to the genesis and progression of HCC cells and works by regulating downstream genes of LOXL2 in certain pathways. Therefore, LOXL2 may play an important role in the progression and prognosis of HCC.

Key words: HCC, LOXL2, SMMC-7721 cell line, RNA interference (RNAi), mechanism analyses.

1. Introduction

A better understanding of hepatocellular carcinoma (HCC)—the most common primary liver cancer, which ranks globally as the third or fourth leading cause of cancer-related death [1, 2], is imperative for improving early diagnosis and subsequent patient treatment and prognosis. Approximately 700,000 new cases are diagnosed each year [3]. HCC frequently leads to poor survival for local invasion and metastasis. Thus, it is important to explore biological target genes for determining treatment. The authors have focused the work on the biological role of lysyl oxidase-like 2 (LOXL2) genes in cancer cells.

Several members of the lysyl oxidase (LOX) family have recently emerged as important regulators of tumor progression [4]. LOXL2 is a member of the LOX gene family, of which the prototypic LOX and LOX-like proteins was encoded from 1 to 4 (i.e., LOXL1, LOXL2, LOXL3 and LOXL4), and has identified in mammalian genomes [5]. The LOX family promotes invasion and metastatic niche formation in many tissues and organs, such as skin, heart, lung, kidney, stomach, colon, ovaries and so on [6, 7]. In recent LOXL2 studies, it was shown that high LOXL2 expression was associated with poor prognosis in the colon, esophageal and squamous cell cancers, and that LOXL2 was correlated closely with tumor invasion and metastasis [8-15]. Whether the LOXL2 gene contributes to the tumorigenicity and progression of HCC has seldom been studied.

In this study, the authors aimed to evaluate the cell cycle and apoptosis in LOXL2-knockdown SMMC-7721 cell line and examine the effects of LOXL2 silencing on the growth of HCC cell lines. Furthermore, they selected downstream genes of LOXL2 by microarray, verified them by Western

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Blotting and then elaborated the downstream genes involved in the biological activities of LOXL2 in the context of HCC. Finally, they achieved the purpose of analyzing a LOXL2 mechanism in the occurrence and development of HCC.

2. Materials and Methods

2.1 Cell Lines and Culture

Human HCC cell lines, i.e., SMMC-7721, HepG2, Hep3B and Huh-7, were obtained from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. Four kinds of cell lines were cultured in Dulbecco's modified eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and antibiotics (100 units/mL penicillin and 0.25 μg/mL streptomycin) (HyClone, Logan, UT, USA). Cells were grown at 37.8 °C in a humidified atmosphere of 5% CO_2 and 95% air.

2.2 Construction of a RNAi Gene with a Lentiviral Vector

Cells expressing scrambled by short-hairpin RNA (shRNA) 5'-TTCTCCGAACGTGTCACGT-3' in a lentiviral vector were used as control, cells transfected with 5'-ATTACTCCAACAACATCAT-3' (LOXL2-siRNA) were used to silence LOXL2, and a human LOXL2 dsDNA oligo was synthesized with targeted siRNA sequences by Genechem Co. Ltd. (Shanghai, China). A lentiviral vector—pGCSIL-GFP plasmid (synthesized by Genechem Co. Ltd., Shanghai) was digested and connected with the dsDNA oligo, and subsequently transformed into competent *E. coli*. Lentiviral vector production and infection were conducted as previously described in Ref. [16]. Stable cell lines expressing LOXL2 shRNAs were selected on Luria-Bertani agar medium after 16 h cultivation at 37 °C and identified by real-time polymerase chain reaction (RT-PCR). The positive clones of recombinant plasmids were sequenced and extracted.

2.3 RT-PCR

Total RNA was extracted from SMMC-7721 cells using Trizol reagent (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. cDNA was obtained by reverse transcription according to the M-MLV operating instructions (Promega, Madison, Wisconsin, USA). Then, RT-PCR was used to detect the mRNA of target gene. The primer sequences: 5'-GTCTGCGGCATGTTTGG-3' and 5'-GCTCTGGCTTGTACGCTTT-3' was for LOX2, and 5'-TGACTTCAACAGCGACACCCA-3' and 5'-CACCCTGTTGCTGTAGCCAAA-3' was for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The step of PCR was 15 s at 95 °C, followed by 45 cycles of 5 s at 95 °C and 30 s at 60 °C. Melting curve analysis was used to check amplification. Data were calculated using the comparative $2^{-\Delta\Delta Ct}$ method.

2.4 Flow Cytometry

2.4.1 Detection of the Cell Cycle

The SMMC-7721 cells infected with lentiviral vector were harvested, centrifuged at 1,200 rpm for 5 min, washed with cold phosphate-buffered saline (PBS) at 4 °C one time and then gathered. Then, the cells were fixed in 70% ethanol and stored at 4 °C for more than 1 h. The cells were centrifuged and washed again, staining solution was added and the cells were resuspended for cell cycle analysis by using flow cytometry (FACSCalibur Instrument, American BD Company). All experiments were carried out in triplicate.

2.4.2 Detection of Apoptosis

The SMMC-7721 cells infected with lentiviral vector were harvested, washed with D-Hanks, and centrifuged at 1,500 rpm for 5 min. The precipitated cells were washed with PBS one time, centrifuged again and collected after being washed with binding buffer. The cell suspension was gathered with a final density of 1×10^6-1×10^7 cells/mL, 5 uL annexin V-APC was added for 10-15 min. Then the cells were transferred for flow cytometry analysis (FACSCalibur

Instrument, American BD Company). The apoptosis kit used in the test was Ebioscience 88-8007. All experiments were carried out in triplicate.

2.5 BrdU Labeling

The SMMC-7721 cells infected with lentiviral vector were plated at a density of 2×10^4/well in 96-well plates, incubated with BrdU reagent (10 uL/well, Roche, Cat. No. 11647229001) and fixed, and the stationary liquid was discarded. Then, substrate solution was added to finish the reaction after staining with anti-BrdU antibodies for 90 min of reaction, and the cells were detected at 24 h and 96 h. A microplate reader was used to measure the absorbance at a wavelength of 450 nm. All experiments were carried in triplicate. Data were calculated, analyzed and showed in a figure.

2.6 Microarray

To select candidate genes for further Western Blotting analysis, the influence of LOXL2 on SMMC-7721 cells infected with siRNA was investigated by microarray analysis. Genome-wide microarray expression profiling was performed using Affymetrix GeneChip® Primeview™ for humans. Microarray experimental procedures were carried out following the manufacturer's protocols. Total RNAs were extracted by Trizol reagent (Invitrogen, San Diego, CA, USA) and qualified with a NanoDrop 2000 (Thremo) and Bioanalyser 2100 (Agilent). Amplified RNA was obtained with a GeneChip® 3'IVT express kit (Affymetrix), hybridized with a GeneChip® probe with a GeneChip® hybridization oven 645 (Affymetrix), washed with a GeneChip® fluidics station 450 (Affymetrix) and scanned. Data were collected and analyzed.

2.7 Western Blotting

Total proteins were extracted using standard radio-immunoprecipitation assay (RIPA) lysis buffer. Protein concentration was determined with a bicinchoninic acid (BCA) protein assay (HyClone-Pierce), and 20 g of protein was loaded in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% gels. Polypeptides were transferred onto polyvinylidene fluoride (PVDF) membranes and blocked with 5% nonfat dry milk. Immunoblots were incubated with the indicated antibodies: rabbit anti-baculoviral IAP repeat-containing proteins (BIRC3) (ab32059, Abcam), 1:500; mouse anti-cell division cycle 42 (CDC42) (ab187643, Abcam), 1:2,000; rabbit anti-FOS (ab7963, Abcam), 1:200; rabbit anti-murine double minute 2 (MDM2) (ab32103, Abcam), 1:200; rabbit anti- transforming growth factor β receptor II (TGFBR2) (ab61213, Abcam), 1:200. Secondary antibodies were anti-rabbit (1:2,000; Santa Cruz) and anti-mouse (1:2,000; Santa Cruz). Bands were visualized using an ECL chemiluminescence kit (Amersham) and X-ray analysis.

2.8 Statistical Analysis

All data are expressed as the mean ± SD. The intergroup difference was compared using a paired student's *t*-test (two tailed). In all cases, values of $P < 0.05$ were considered statistically as significant. These analyses were carried out using the IBM SPSS version 19.0 software.

3. Results

3.1 LOXL2 Expressed in Different Human HCC Cell Lines

RT-PCR showed that LOXL2 mRNA was significantly up-regulated in four types of HCC cell lines, particularly in HepG2 and SMMC-7721 cells (Fig. 1).

3.2 Expression of LOXL2 mRNA Decreased in SMMC-7721 Cell Line with LOXL2-siRNA

The RT-PCR results showed that the expression of LOXL2 mRNA was decreased in SMMC-7721 cell line ($P = 7.28827E - 08$, $P < 0.01$), which was

infected with LOXL2-siRNA (Fig. 2). It is suggested that the lentiviral vector played an effective role in LOXL2 gene silencing.

3.3 LOXL2 Silencing Induced Cycle Arrest in HCC Cells

When compared with the control, LOXL2 silencing dramatically decreased the fraction of G1 phase (P = 0.000, $P < 0.001$), and increased the percentage of S-phase (P = 0.06, $P < 0.001$) in SMMC-7721 cells (Fig. 3). It indicated that LOXL2 contributed to the cell phase transition of HCC cells.

3.4 LOXL2 Silencing Increased Apoptosis in HCC Cells

After LOXL2 being silenced, the percentage of apoptosis was increased in SMMS-7721 cells (P = 5.63882E – 06, $P < 0.01$) (Fig. 4) compared with the controls. The result revealed that LOXL2 silencing increased apoptosis of HCC cells.

3.5 LOXL2 Silencing Inhibited HCC Cell Growth

BrdU labeling was used to detect the absorbance folds of infected SMMC-7721 cells due to thymine

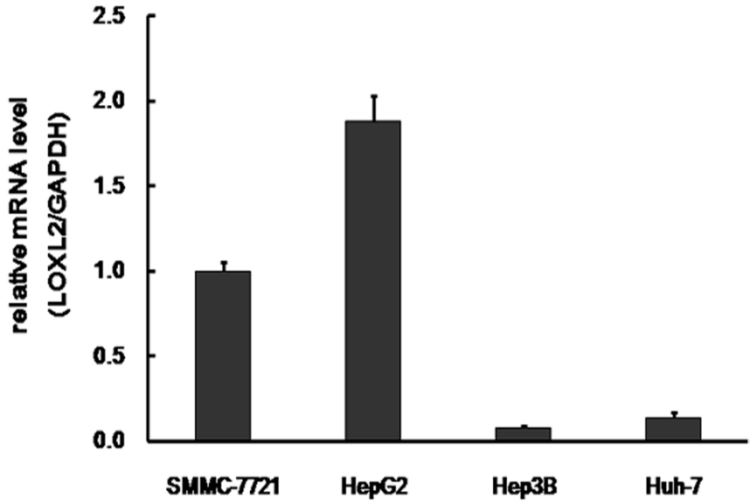

Fig. 1 Expression of the LOXL2 in four HCC cell lines.

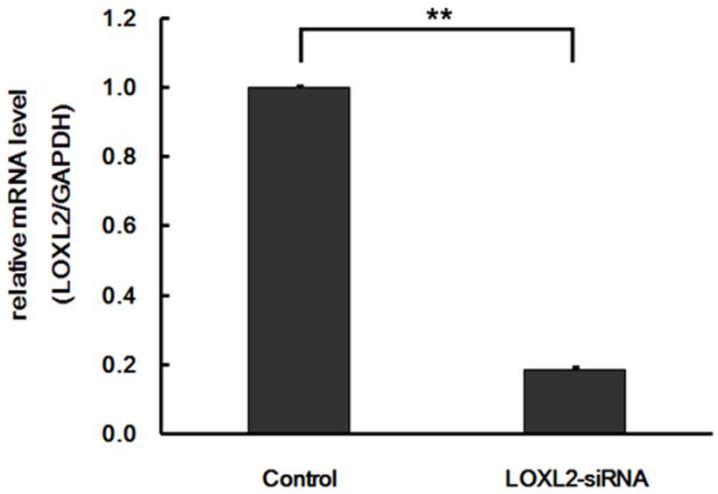

Fig. 2 Comparison of LOXL2 mRNA expression in SMMC-7721 cell lines with LOXL2-siRNA and controls.
**Means the differences between LOXL2-siRNA and control are at level of $P < 0.01$.

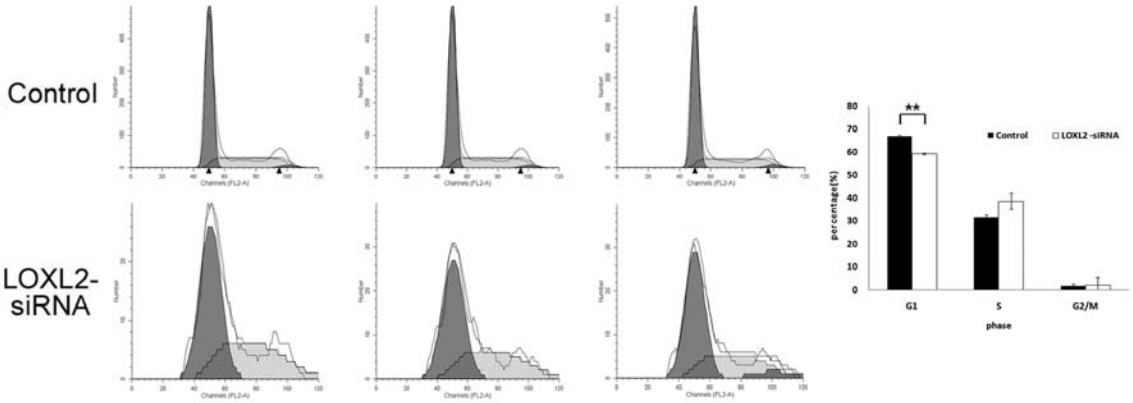

Fig. 3 Effects of LOXL2 silencing on SMMC-7721 cell cycle distributions.
Cell cycle analysis of SMMC-7721 cell line using flow cytometry and data showing percentage of different cell cycle phases. **Means the difference between LOXL2-siRNA and control are at level of $P < 0.01$.

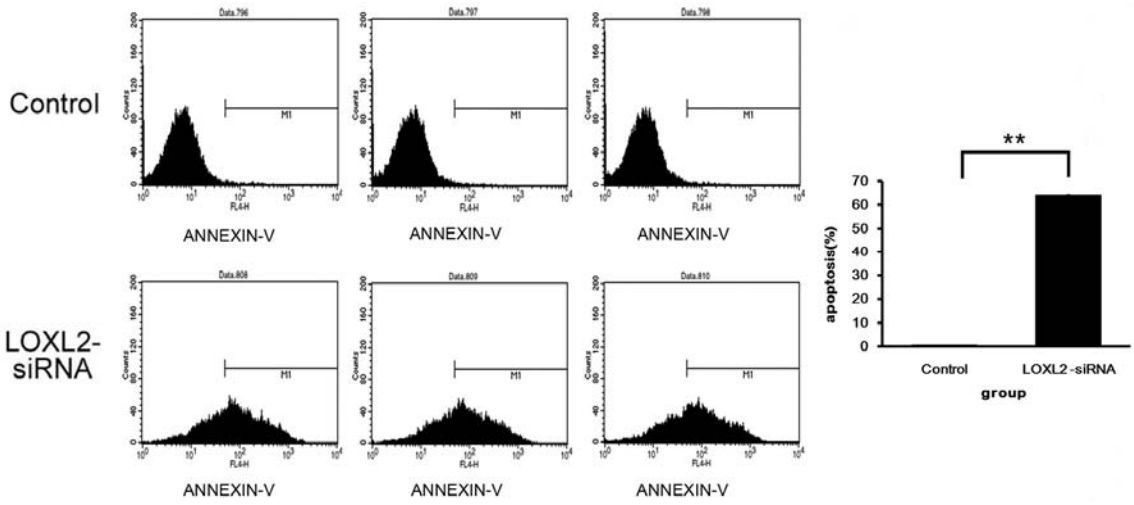

Fig. 4 Effects of LOXL2 silencing on SMMC-7721 cell apoptosis.
Cell apoptosis analysis of SMMC-7721 cell line using flow cytometry and data showing apoptosis rate. **Means the difference between LOXL2-siRNA and control are at level of $P < 0.01$.

replacement competition in the sequence in S phase, and it was found that LOXL2 silencing decreased the growth of SMMC-7721 cells on the reference days ($P = 0.015068162$, $0.01 < P < 0.05$) (Fig. 5). The results indicated that LOXL2 played a significant role in suppressing the growth of HCC cells *in vitro*.

3.6 Downstream Genes of LOXL2 Selected by Microarray in HCC Cells

Based on the screening criteria of an absolute fold change value > 1.5 and P value < 0.05, a total of 1,529 differentially expressed genes (DEGs) were identified in the control and LOXL2 silenced cells, in which there were 637 up-regulated genes and 892 down-regulated genes. The expression of LOXL2 was down-regulated ($P = 3.21E - 06$, $P < 0.01$) after silencing. Subsequently, the DEGs were subjected to pathway enrichment analysis according to the Kegg and BioCarta information and ranked by P value, and the top 10 are shown in Fig. 6. At the same time, the DEGs were subjected to gene ontology analysis according to the differences in gene functions. Then, LOXL2 was added into the first pathway banding according to the significant probability value that was the lowest ($P = 2.59E - 09$, $P < 0.01$), and a gene network diagram was constructed (Fig. 7). In conclusion, it was speculated that the target gene LOXL2 worked in HCC by regulating the MDM2,

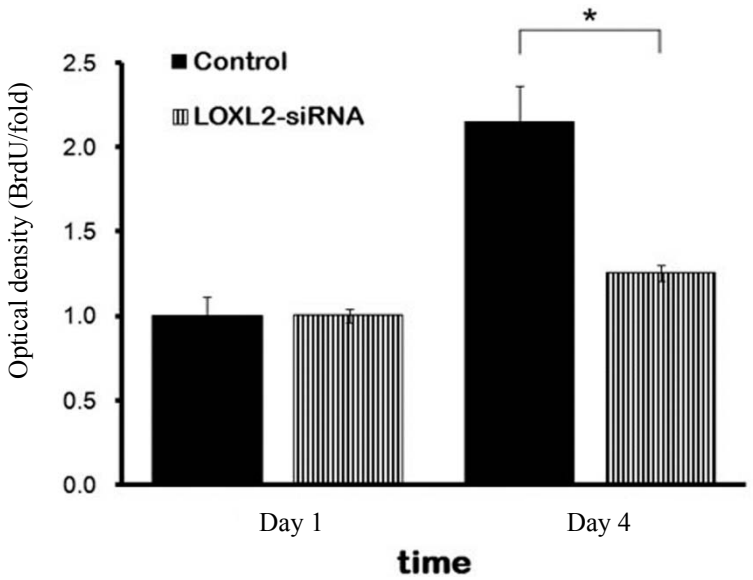

Fig. 5 Effects of LOXL2 Silencing on SMMC-7721 cell absorbance.
BrdU labeling was performed on the indicated days to show the absorbance of SMMC-7721 cells. *Means the difference between LOXL2-siRNA and control are at level of $0.01 < P < 0.05$.

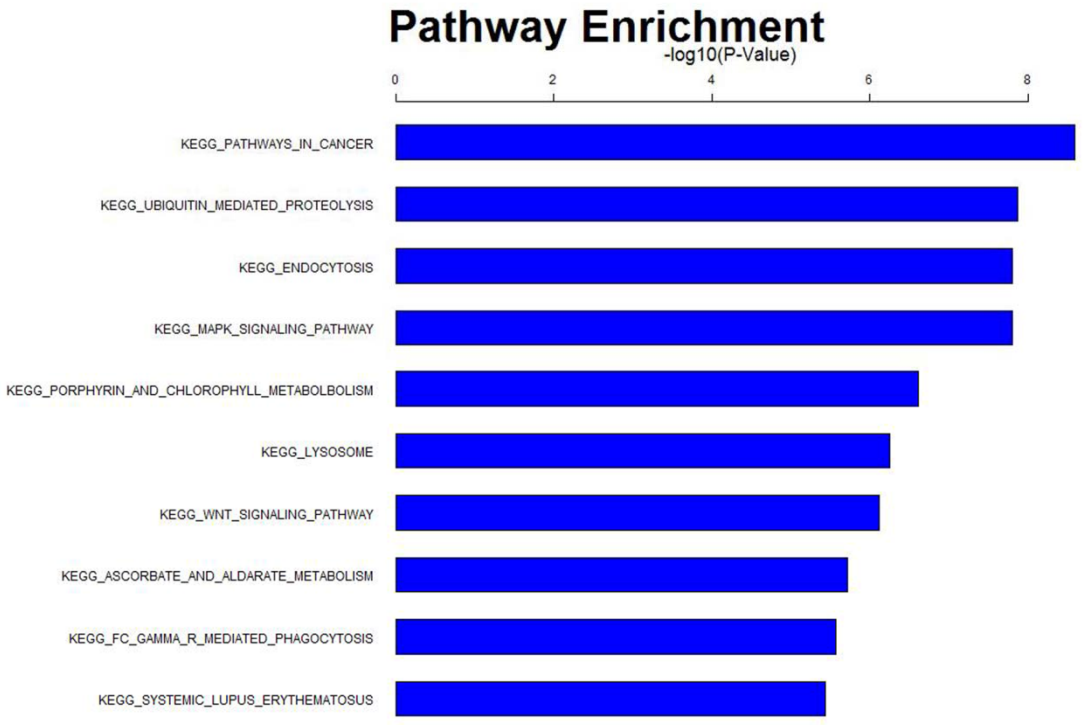

Fig. 6 Rankings of the enrichment pathway of DEGs.

BIRC3, CDC42, FOS and TGFBR2 genes.

3.7 MDM2, BIRC3, CDC42, FOS and TGFBR2 Verified by Western Blotting

As shown in Fig. 8, MDM2, BIRC3, CDC42, FOS and TGFBR2 genes were identified by Western Blotting. Among them, the expressions of BIRC3, MDM2 and TGFBR2 were decreased. On the contrary, CDC42 expression was increased, and there was no change in FOS expression. Therefore, the results indicated

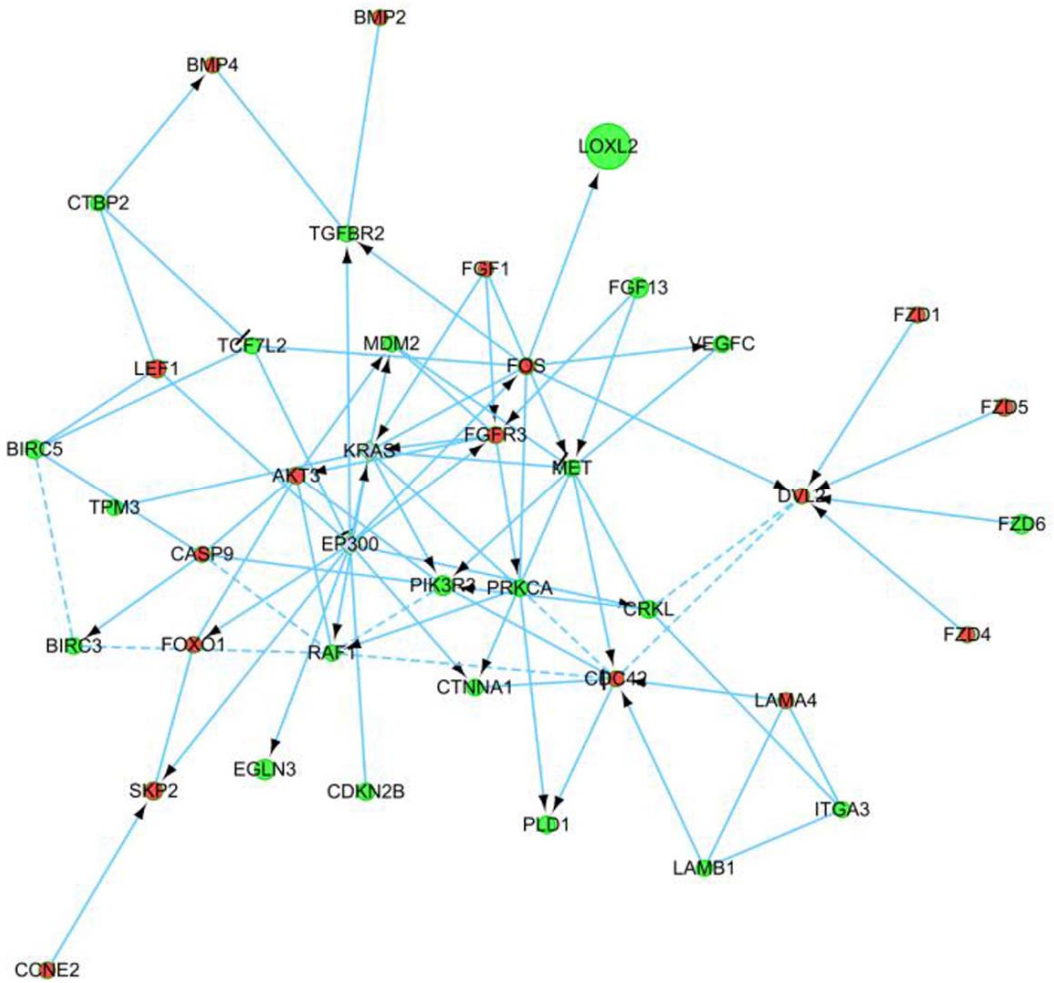

Fig. 7 Interaction network of the first pathway banding.

Red and blue nodes represent up- and down-regulated genes, respectively. The solid arrow represents the relationship with LOXL2, and the dotted line predicted the relationship; the "T" represents the inhibitive effect.

that in the "Kegg pathways in cancer", LOXL2 silencing down-regulated the expression of BIRC3, MDM2 and TGFBR2, up-regulated the expression of CDC42, and had no obvious effect on FOS.

4. Discussion

The present studies showed that the expanded expression of LOXL2 can promote tumor invasion and metastasis [8-15]. Nevertheless, the function of LOXL2 in HCC has not yet been elucidated. In the study, LOXL2 mRNA had high expression levels in four types of HCC cell lines, particularly in HepG2 and SMMC-7721 cells. Because the sources of HepG2 and SMMC-7721 cells are different, the authors choose SMMC-7721 cell line for further research. Additionally, the expression of LOXL2 mRNA was decreased in SMMC-7721 cell line infected with siRNA lentiviral vector. In parallel, the results provide new evidence that silencing LOXL2 expression could increase the rate of cell apoptosis and decrease the growth of HCC cells *in vitro*. Moreover, in the cell cycle distribution of SMMC-7721, LOXL2 silencing dramatically decreased the fraction of G1 phase and increased that of S phase; it indicated that LOXL2 contributes to HCC cell cycle distribution. If we artificially interfere in cell cycle phase, HCC growth maybe blocked. In the future, it will be beneficial in the clinical treatment of HCC patients.

Although it is known that LOXL2 can promote tumorigenicity and progression in HCC, many problems

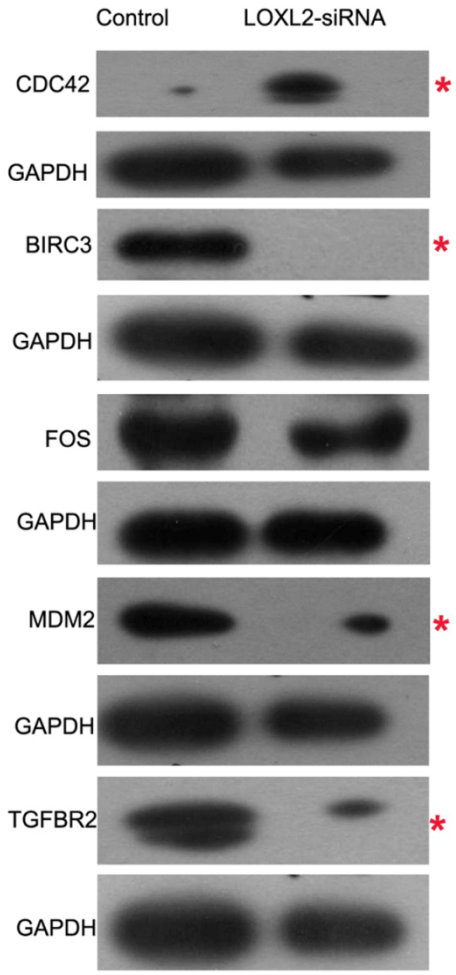

Fig. 8 Verification of MDM2, BIRC, CDC42, FOS and TGFBR2 expression by Western Blotting.
*Means $0.01 < P < 0.05$.

about this process remain unsolved, such as those regarding the regulatory mechanism of this process. Here, it was shown that the target gene LOXL2 worked in HCC by regulating the MDM2, BIRC3, CDC42 and TGFBR2 genes through microarray analysis. According to the results, LOXL2 up-regulated BIRC3, MDM2 and TGFBR2 genes, and down-regulated CDC42 significantly.

CDC42, which is a small GTPase belonging to the Rho subfamily, plays multiple roles in cellular functions, including cell proliferation, migration and apoptosis, and it even promotes malignant transformation [17-22]. It was originally identified in *S. cerevisiae* as a mediator of cell division [23, 24]. Since its original discovery, CDC42 has been found to influence a variety of signaling events and cellular processes [25, 26]. The up-regulation of CDC42 is involved in cell polarity and cell movement [26, 27]. Previous studies have also demonstrated that CDC42 played a crucial role in the transforming growth factor (TGF) pathway [28]. Additionally, as a member of the transforming growth factor β family, TGF-β works as a multi-functional cytokine and plays an important role in cell proliferation, apoptosis and differentiation [29]. TGF-β is an important gene that has been identified as a cancer susceptibility gene that exerts tumor-suppressive effects that cancer cells must elude for malignant evolution [29-32]. TGFBR1 and TGFBR2—two transmembrane serine/threonine kinase receptors, are required for TGF-β signaling transduction. Herein, it was demonstrated that when HCC occurs, LOXL2 exerts its effect by down-regulating the CDC42 gene and up-regulating the TGFBR2 gene. This result hinted that the target gene may be useful in tumor therapy.

MDM2 is an intracellular molecule with multiple biological functions that negatively regulate the tumor suppressor p53 [33]. The expression of MDM2 is linked to gain-of-function mutations in many tumors [34]. In addition, the inhibition of MDM2 was shown to block tumor growth or induce cell apoptosis in a number of tumors [35-39]. In addition, DNA amplification of BIRC3 has been observed in mouse liver and human lung cancers, liver carcinoma, oral squamous cell carcinoma, medulloblastoma, glioblastoma and pancreatic cancer [40]. In this study, the expression levels of MDM2 and BIRC3 were significantly lower in HCC cells with LOXL2 silencing, which means that LOXL2 up-regulated MDM2 and BIRC3 in HCC. A larger prospective study is needed to determine the exact roles of CDC42, TGFBR2, MDM2 and BIRC3 genes and their complex linkage in HCC.

5. Conclusions

In summary, LOXL2 contributes to the genesis and

progression of HCC cells and works by regulating downstream genes of LOXL2 in certain pathways. Therefore, LOXL2 may play an important role in the progression and prognosis of HCC. Furthermore, additional studies will be required to fully understand the molecular mechanisms involved.

Acknowledgments

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